

**INVESTIGATING THE ROLE AND ACTIVITY OF CC-TYPE  
GLUTAREDOXINS IN THE REDOX REGULATION OF  
TGA1/TGA4 IN *ARABIDOPSIS THALIANA***

**A Thesis Submitted to the  
College of Graduate Studies and Research  
In Partial Fulfillment of the Requirements for the**

**Degree of Masters Science  
In the Department of Biology,  
University of Saskatchewan  
Saskatoon, Saskatchewan, Canada**

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## **Acknowledgements**

I would like to thank my research supervisor Dr. Pierre Fobert for taking the time to take me on as a graduate student. His patience and guidance has been invaluable and I have learned an immense amount in such a short period of time. In particular I would like to express my gratitude to him throughout the writing process, as I found this the most challenging part of my masters program. For his support and encouragement I would again like to say thank you.

I would also like to thank my supervisory committee: Dr. D. Sanders and Dr. Y. Wei. Their feedback, patience, and encouragement throughout my project were very much appreciated. I would also like to thank Kevin Rozwadowski for taking the time to be my external examiner for the defense.

The members of the Fobert lab were not only helpful in teaching lab techniques and offering sound advice but many became good friends as well. I would like to especially thank Heather Shearer, Marc Champigny, Nirmala Sharma, Shobha Devi, Catherine DeLong, Maureen Anderson, and Lipu Wang. Their guidance has been precious.

Finally I would like to thank all the people who stood by me outside of work. My family and friends have been a great source of encouragement and love. They were always available for much needed support. I would especially like to thank my parents, to whom I am indebted forever for all they have done for me.

This project was supported by a NESERC Discovery grant to Dr. P. Fobert, and the University of Saskatchewan.

## **Abstract**

Plants respond to and defend themselves against a wide range of disease-causing microbes. In order to do so, massive reprogramming of cellular protein expression patterns, which underpin various defense pathways, must occur. A family of basic leucine zipper transcription factors, called TGA factors, has been implicated in mediating this response. The TGA factors themselves are subject to complex regulation; of note, TGA1 and TGA4 are regulated via a reduction of conserved cysteines after treatment with the phenolic signaling molecular salicylic acid, which accumulates following pathogen challenge. Previous studies indicate that TGA factors physically interact in the yeast two-hybrid system with the plant-specific CC-type of glutaredoxin (Grx)-like proteins. Grx are a family of oxidoreductases that are important for maintaining the cellular redox status and often are required to modulate protein activity. The goal of this study was to ascertain the role of these Grx-like proteins in regulating TGA1 redox state. To this end, the expression patterns of several Grx genes were analyzed.

Quantitative-reverse-transcriptase PCR (q-RT-PCR) experiments indicated that TGA1 and TGA4 may be involved in down-regulating levels Grx-like gene transcripts after exposure to pathogens or salicylic acid (SA). Furthermore, qRT-PCR experiments also indicated that expression of some Grx-like genes is induced by SA, jasmonic acid (JA), and *Pseudomonas syringae*. Overexpression of the Grx-like protein, CXXC9, in *Arabidopsis thaliana* revealed that it is a regulatory factor in the cross-talk between

the SA/JA pathways as it is able to suppress expression of PDF1.2, a marker for the JA defense pathway, as determined by qRT-PCR. The  $\beta$ -hydroxy ethyl disulfide (HED) assay was utilized to determine if the CC-type of Grx-like proteins have oxidoreductase activity *in vitro*. These studies revealed that that the Grx-like proteins do not exhibit oxidoreductase activity in this assay.

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## List of Abbreviations

<b>2YT</b>	2X yeast, tryptone
<b>° C</b>	degrees centigrade
<b>%</b>	percentage
<b>μ</b>	micro-
<b>A</b>	absorbance
<b><i>as-1</i></b>	activating sequence 1
<b>BME</b>	β-mercaptoethanol
<b>BSA</b>	bovine serum albumin
<b>BTB/POZ</b>	broad-complex, tramtrack, and bric-a-brack/pox virus and zinc finger
<b>BTH</b>	benzol (1, 2, 3) thiadiazole-7-carbothionic acid S-methyl ester
<b>bZIP</b>	basic leucine zipper
<b>CaMV</b>	Cauliflower Mosaic Virus
<b>cDNA</b>	complementary DNA
<b>CFU</b>	colony forming units
<b>Cys</b>	cysteine
<b>DNA</b>	deoxyribonucleic acid
<b>dNTP</b>	2'-deoxynucleotides 5'-triphosphate
<b>DTT</b>	dithiothreitol
<b>EDTA</b>	ethylenediamine-tetraacetic acid
<b>g</b>	gram
<b>GST</b>	glutathione S-transferase
<b>GSH</b>	reduced glutathione
<b>GSSG</b>	oxidized glutathione

<b>HR</b>	hypersensitive response
<b>h</b>	hour
<b>INA</b>	2,6-dichloroisonicotinic acid
<b>IPTG</b>	isopropyl- $\beta$ -D-thiogalactopyranoside
<b>ISR</b>	induced systemic resistance
<b>JA</b>	jasmonic acid
<b>l</b>	litre(s)
<b>LS</b>	linker scan
<b>m</b>	mili-, meter(s)
<b>M</b>	molar
<b>min</b>	minute(s)
<b>mol</b>	mole(s)
<b>mRNA</b>	messenger RNA
<b>n</b>	nano-
<b>NIM1</b>	non-inducible immunity 1
<b>Ni-NTA</b>	nickel-nitrilotriacetic acid
<b>NPR-1</b>	non-expresser of pathogenesis-related-1
<b>OD</b>	optical density
<b>PCR</b>	polymerase chain reaction
<b>pH</b>	percentage of hydrogen
<b>PR-1</b>	pathogenesis-related-1
<b>RNA</b>	ribonucleic acid
<b>ROS</b>	reactive oxygen species
<b>rpm</b>	revolutions per minute

<b>p.v.</b>	pathovar
<b>SA</b>	salicylic acid
<b>SAR</b>	systemic acquired resistance
<b>Ser</b>	serine
<b>SDS</b>	sodium dodecyl sulphate
<b>SDS-PAGE</b>	sodium dodecyl sulphate- polyacrylamide gel electrophoresis
<b>sec</b>	second
<b>TE</b>	Tris buffer and EDTA
<b>U</b>	unit(s)
<b>V</b>	volts

## **Chapter 1: Introduction**

In the face of the onslaught of plant/pathogen interactions, plants have evolved sophisticated mechanisms to thwart potential pathogens. One such mechanism is the preformed or innate defenses consisting of, but not limited to, cell wall, cuticle, and antimicrobial compounds (Mysore and Ryu, 2004). The second is the induced responses which are stimulated upon the pathogens successful avoidance of the innate defenses and/or recognition at the plant cell wall (Mysore and Ryu, 2004). The hallmark of the induced responses is the ability to regulate hundreds of genes in a coordinated and timely fashion (Euglem, 2005).

The massive reprogramming of cellular machinery is partially coordinated by upstream signaling molecules and proteins. These molecules are responsible for the post translational modifications, including oxidation/reduction, phosphorylation, and ubiquitination, to name a few, of transcription factors thereby regulating their activity. The ultimate goal of the induced responses is to create a long lasting, broad spectrum resistance to pathogens through for example, reinforcement of physical barriers, activation of programmed cell death and induction of antimicrobial proteins and molecules (Mysore and Ryu, 2004).

Further identification of transcription factor involved in defense, their targets, and their regulatory mechanisms is essential to understanding their role in the defense response. Thus far only a small portion of the defense pathway has been studied: while many of the transcription factors involved have been identified, there is still much to be

learned with regards to their regulation and potential targets (Euglem, 2005). Understanding of the transcriptional regulation and targets will be indispensable in facilitating further study of key genes and mechanisms in the defense response.

## **Chapter 2: Literature Review**

### **2.1 Plant Defense Responses**

Plants are constantly exposed to a wide range of microbes, some of which have evolved mechanisms to become disease-causing agents (i.e. pathogens). In response, plants have evolved numerous ways to cope with the onslaught of pathogens and potential pathogens. These mechanisms are of two types: innate or constitutive, and inductive. Examples of constitutive defenses include the cell wall, waxy cuticle, as well as proteins and secondary metabolites possessing direct antimicrobial properties. Inducible defense responses include reinforcement of physical barriers, programmed cell death at the site of infection called the hypersensitive response (HR), production of reactive oxygen species (ROS), induction of antimicrobial secondary metabolites known as phytoalexins, and induction of pathogenesis-related (PR) genes and proteins (Mysore and Ryu, 2004). Interestingly, recognition of pathogens that do not readily cause disease results in a more rapid and intense inducible defense response than seen in reaction to disease-causing pathogens (Euglem, 2005).

There are two main types of microbial factors, called elicitors, which are capable of inducing a defense response: pathogen associated molecular patterns (PAMPs) and avirulence factors. PAMPs are structures or molecules possessed by the pathogen or are byproducts necessary to its lifestyle. The former being highly conserved across many microbial species. Examples of PAMPs include: lipopolysaccharide (LPS) on Gram negative bacteria; flagellin, the protein component of the flagellum; and major



components of fungal cell walls such as chitin or ergosterol (Chrisholm et al., 2006; Ellis, 2006). These components activate a resistance mechanism known as PAMP-

triggered immunity (PTI) which relies on the inducible defenses mentioned above. PTI is very highly conserved among members of a particular plants species, and is effective against a broad spectrum of pathogens (Chrisholm et al., 2006). It is largely responsible for a very stable type of disease resistance known as non-host resistance.

Microbes have evolved mechanisms to bypass PTI by producing molecules called effectors, which act as virulence factors to mask the presence of the pathogen and/or disable components of the non-host defense machinery (Bonas and Lahaye, 2002; Ellis, 2006). In these cases plants are still able to mount a residual defense response, sometimes referred to as basal resistance. Basal resistance does not fully protect the plant from pathogens, although it slows pathogen growth and limits spread (Euglem, 2005).

Plants have evolved ways to recognize pathogen virulence factors through the action of *Resistance*-genes (*R*-genes). In such cases, the virulence factor becomes a so-called avirulence factor and elicits a form of resistance called effector triggered immunity (ETI) (Chrisholm et al., 2006; Jones and Dangl 2007). Possession of an avirulence factor causes a normally virulent pathogen to become avirulent on plant genotypes possessing the corresponding *R*-gene. *R*-gene products are typically receptor type molecules capable of transducing a signal and interact either directly with avirulence factors or act through an indirect mechanism such as a co-receptor type molecule (Bonas and Lahaye, 2002). Recognition of avirulence factor leads to the triggering of ETI (Bonas and Lahaye, 2002; Jones and Dangl, 2007). Unlike the

conserved PAMPs, avirulence factors are highly polymorphic within a pathogen population; likewise *R*-genes are also highly polymorphic across a specific plant species (Mysore and Ryu, 2004; Chrischold et al., 2006). As a result ETI is an example of host resistance where both resistance and susceptibility to a particular pathogen species can be identified within a single plant species.

A secondary consequence of both ETI and PTI is the establishment of broad range and long-lasting resistance to otherwise virulent pathogens known as systemic acquired resistance (SAR) (Hunt and Ryals, 1996; Durrant and Dong, 2004). Other types of induced resistance, mechanistically distinct from SAR, have also been characterized. These include induced systemic resistance (ISR, which is triggered by certain rhizobacteria (Beckers and Spoel, 2006). Both SAR and ISR stem from a complex web of signaling from the elicitation event occurring at or near the cell surface of the plant.

## **2.2 Role of Plant Defense Hormones**

The defense responses described in the previous section are typically elicited by biotrophic pathogens (i.e. those that derive nutrients from live plant tissue) (Beckers and Spoel, 2006). Such interactions are associated with the rapid accumulation of the phenolic signaling molecule salicylic acid (SA) and the expression of PR genes and proteins in the plant (Durrant and Dong, 2004). Treating plants topically with SA, or SA analogues such as 2,6-dichloroisonicotinic acid (INA) or benzothiadiazole S-methyl ester (BTH), also leads to enhanced resistance to virulent biotrophic pathogens, sometimes referred to as chemical SAR. Furthermore, transgenic plants that express

bacterial *NahG*, encoding a salicylate hydroxylase that degrades SA to catechol, and mutants compromised in SA biosynthesis fail to accumulate SA and PR genes, and fail to mount a defense response (Durrant and Dong, 2004).

Plants may also be susceptible to infection by necrotrophs, which derive nutrient from dead tissue (Beckers and Spoel, 2006). Signaling in response to necrotroph challenge is dependent on the gaseous plant hormone ethylene (C<sub>2</sub>H<sub>4</sub>) and oxylipins, also known as jasmonates, the most prominent one being jasmonic acid (JA) (Durrant and Dong, 2004; Beckers and Spoel, 2006). ISR also requires intact ethylene and oxylipin signaling pathways, while mechanical wounding and herbivore feeding rely on oxylipin signaling (Beckers and Spoel, 2006). Depending on the type of pathogen or stress inflicted on the plant, different jasmonates can be produced leading to a finely tuned defense response that will regulate different sets of genes (Beckers and Spoel, 2006).

Because of their different signaling requirements, it was initially thought that SAR was antagonistic to ISR, with SAR being the more dominant response (Beckers and Spoel, 2006). However, recent findings point to a synergism between the SAR and ISR pathways. Studies indicate that simultaneous induction of SAR and ISR results in a more enhanced resistance to a virulent strain of the bacterial pathogen *Pseudomonas syringae* than either response alone (Becker and Spoel, 2006; Truman et al., 2007). Another study indicated that the accumulation of JA preceded the accumulation of SA in response to bacterial infection, further demonstrating that JA, ethylene and SA are dependent on one another for induction of a sustained resistance response (Becker and Spoel, 2006; Truman et al., 2007).

There is still solid evidence that SA and JA signaling pathways are mutually antagonistic; such an instance was reported in studies with plants containing a mutation in CORONATINE INSENSITIVE1 (COI1), which regulates the synthesis of JA (Becker and Spoel, 2006). These plants were found to be more resistant to bacterial pathogens due to an increase in SA accumulation and induction of SAR, thus demonstrating JA's antagonistic effect on SA accumulation. Furthermore, plants infected with a virulent strain of the bacterial pathogen *P. syringae* that produces a JA mimic (the phytotoxin coronatine) were found to have reduced resistance to bacterial infection likely due to JA-induced suppression of SAR (Becker and Spoel, 2006). In contrast, SA can also be a potent inhibitor of JA-induced ISR. For example, ISR can be nullified by prior triggering the SAR response indicating that SA may be suppressing the JA-inducible response (Becker and Spoel, 2006). Cross-talk between JA/C<sub>2</sub>H<sub>4</sub> and SA and their associated induction of the related resistance response still needs further study to fully understand the interactions between the two pathways.

### **2.3 Transcriptional Regulation During Plant Defense Responses**

The induction of plant defense responses results in re-programming of the plant transcriptome, in particular the induction of the *PR* genes (Euglem, 2005). These genes have been found in a wide range of plant species and encode proteins with diverse antimicrobial functions chitinases,  $\beta$ -1, 3-glucanases, proteinase-inhibitors, defensins and peroxidases (Van Loon and Van Strien, 1999). There are at least 14 families of PR proteins that work collectively to inhibit pathogen growth and spread (Van Loon and Van Strien, 1999). Some of these PR genes and proteins, in particular PR-1, PR-2 and

PR-5, are often used as markers to monitor SAR induction (Ryals et al., 1996). In addition to *PR*-genes, a multitude of other genes encoding proteins involved in every aspect of plant physiology are differentially expressed in response to pathogen challenge. It is evident that the coordination and timing of transcription of these defense genes are essential to the state of plant disease resistance (Euglem, 2005; Fobert, 2006).

The exquisite control of transcription is carried out by proteins called transcription factors. Transcription factors can be categorized into three broad types: basal transcription factors which are required for binding and activity of RNA polymerase; sequence-specific transcription factors that bind to specific sequences in the promoters of particular genes or gene families and serve to recruit (directly or indirectly) the basal transcriptional machinery to these promoters; and cofactors that modulate the activity and/or binding capacity of transcription factors (White, 2001). Thus, transcription of genes is determined by the collection of *cis*-acting regulatory elements contained within a gene and the presence of active *trans*-activating factors (transcription factors) in combination (Fobert, 2006). A transcription factor can accordingly be activated by the signals transduced as a result of pathogen recognition (Fobert, 2006).

### *2.3.1 Transcriptional Co-Factors: NPR1*

NONEXPRESSOR OF PATHOGENESIS-RELATED GENES1 (NPR1) is the primary example of a transcriptional co-factor, a protein that is required for gene transcription without binding directly to DNA, involved in plant defense regulation (Durrant and Dong 2004). Analysis of loss-of-function mutations at the *NPR1* locus has revealed it to be a central regulator of the various types of resistance such as: ETI

mediated through a subset of *R*-genes, basal resistance, SAR, and ISR (Bonas and Lahaye, 2002; Dong, 2004). This loss of resistance is associated with the failure to express numerous genes in response to pathogen challenge or treatment with SA, including *PR-1*, *PR-2* and *PR-5* (Cao et al., 1994; Delaney et al., 1995; Pan et al., 2004; Wang et al., 2005; Mosher et al., 2006).

Further study has revealed that *NPR1* is constitutively expressed and its expression is only slightly higher after treatment with SA (Dong, 2004). Transgenic overexpression of NPR1 results in enhanced resistance to certain pathogens. However, *PR* gene expression remains dependent on elicitation by pathogen or SA-treatment of the transgenic plants, indicating post-translational modification may be involved in the activation of the NPR1 protein (Durrant and Dong, 2004). NPR1 is devoid of any known DNA-binding domain and is thought to regulate *PR* gene expression indirectly through its interaction with sequence-specific transcription factors.

### *2.3.2 Transcription Factors Involved in Mediating Defense Responses*

There are several families of transcription factors that have members involved in the regulation of defense-related genes. These include the WRKY, Ethylene Response Factors (ERFs), Whirly, Myb, and TGA factors (Euglem, 2005; Fobert, 2006). Each of these transcription factors has a distinct structure and role in mediating events in the plant's defense response. Only the TGA factors will be considered here.

### 2.3.3 TGA Family of Transcription Factors

TGA factors belong to the basic leucine zipper (bZIP) family of transcription factors (Ramachandran et al., 1994). These factors contain a basic DNA binding domain that binds to a TGACG sequence (hence the name TGA) and a leucine zipper dimerization domain that form homo- or hetero-dimers (Ramachandran et al., 1994). TGA factors were isolated due to their ability to bind to and activate transcription in roots of tobacco plants from the *activating sequence 1 (as-1)* element of the *Cauliflower Mosaic Virus* 35S promoter (Katagiri et al., 1989). There are ten TGA family members in *Arabidopsis thaliana*, which have been further subcategorized into sub-families on the basis of sequence similarity. The basic region and leucine zipper region are highly conserved among all family members. Only the N-terminal region has notable dissimilarity, indicating that these subfamilies may have distinct regulatory features (Xiang et al., 1997).

The *as-1* and related *cis*-acting elements have been found in the promoters of *PR-1* and *glutathione S-transferase (GST)* genes from *Arabidopsis* and tobacco, and have been implicated in responsiveness to the growth regulators SA, JA and auxin (Xiang et al., 1996; Lebel et al., 1998; Pontier et al., 2001). A mutation of the TGA factor binding-element, called linker-scan 7 (*LS7*), in the *Arabidopsis PR-1* promoter abolishes SA-mediated induction of this gene (Lebel et al., 1998).

Demonstration of the TGA factors' physical interaction with NPR1 implicated them as potential mediators of plant defense genes (Zhang et al., 1999; Després et al., 2000; Niggeweg et al., 2000; Zhou et al., 2000). NPR1 interacts differentially with the members of the TGA family: TGA2, TGA3, TGA5, TGA6 and TGA7 all appear to bind

NPR1 with varying degrees of affinity in yeast and *in vitro* (Després et al., 2000; Zhou et al., 2000). NPR1 does not interact with PERIANTHIA (PAN), TGA1 or TGA4 (see discussion below on TGA1 and TGA4) and has not been tested against the remaining two members of the TGA family. Further support for the interaction of TGA factors with NPR1 comes from an *in vivo* experiment using a protein fragment complementation assay. In this study the interaction between TGA2 and NPR1 was found to occur only after treatment with SA, and to be predominantly localized to the nucleus (Subramaniam et al., 2001). Mutations in NPR1 that compromise *PR* gene expression and disease resistance also disrupt the physical interactions of NPR1 with known interacting TGA factors, suggesting that these protein-protein interactions are of biological relevance.

Additional *in vitro* studies revealed that interaction with NPR1 increased the binding of TGA2 and TGA3 to their cognate *cis*-acting elements, including *as-1* and the *PR-1 LS7* (Després et al., 2000). Similarly, the *in vivo* DNA-binding activities of TGA2 and TGA3 and transactivation properties of TGA2 are compromised in the *npr1* mutants (Fan and Dong, 2002; Johnson et al., 2003). These studies also demonstrated that SA-induction was required for TGA function. In contrast, a recent study demonstrated that neither SA nor NPR1 are required for *in vivo* binding of TGA2 to the *PR-1* promoter (Rochon et al., 2006). Results of Rochon et al. (2006) also revealed that TGA2 is not a transcriptional activator of *PR-1* in either resting or SA induced cells, but rather is a transcriptional repressor in the absence of NPR1. However, in SA stimulated cells NPR1, in conjunction with TGA2, is recruited into an activating complex called an



enhanceosome that requires the BTB/POZ domain and oxidation of two C-terminal Cys residues of NPR1 in order to activate transcription of *PR-1* (Rochon et al., 2006).

Most of the studies on TGA factors have focused on the *Arabidopsis* TGA2 clade, containing TGA2, TGA5, and TGA6 (Xiang et al., 1996). Analysis of dominant-negative TGA factor transgenes, encoding proteins that cannot bind to DNA but retain the potential to dimerize with endogenous protein partners, revealed that TGA factors may have both positive and negative regulatory roles in regulating *PR* genes and defense responses (Pontier et al., 2001). In one report, a phenotype similar to that of the *npr1* mutant was observed (Fan and Dong, 2002). Simultaneous mutation of *tga2*, *tga5*, and *tga6* also gave a phenotype similar to the *npr1* mutant (Zhang et al., 2003). Neither the *tga6* single mutant, nor a *tga2*, *tga5* double mutant were found to be compromised in defense responses. Furthermore, the triple mutant was functionally complemented by transgenic expression of *TGA2* (Zhang et al., 2003). Together, these observations argue that the members of the TGA2 clade are essential functionally redundant mediators of plant defense responses. A recent study (Kesarwani et al. 2007) indicates that the functional differences within the TGA2 clade of *Arabidopsis* transcription factors may be more complicated than previously thought. These authors reported on a new loss-of-function allele of *tga2* (*tga2-2*) that leads to increased expression of *PR-1* in the presence of functional *TGA5* and *TGA6*, suggesting a role for TGA2 as a negative regulator of *PR* gene expression and possible non-redundant functionality.

#### 2.3.4 *TGA1 and TGA4*

Factors in the TGA1 clade (or clade I), TGA1 and TGA4, were not initially found to interact with NPR1 in yeast, but later discovered to require post-translational

modification (PTM) in order to interact (Després et al., 2000; Després et al., 2003). Chromatin immunoprecipitation (ChIP) with tobacco TGA1a, which may be orthologous with *Arabidopsis* TGA1, revealed this transcription factor binds to the promoters of *GST* genes in response to xenobiotic stress treatment (Johnson et al., 2001). Additionally, RNA interference-mediated suppression of *TGA4* stimulated the SA- and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) - induced expression of a transgenic reporter gene under the control of tandemly-repeated *as-1* elements (Foley and Singh, 2004). These results implicate TGA1 and TGA4 with putative roles in response to SA and oxidative stress. Further evidence for a role in mediating defense responses comes from viral induced gene silencing (VIGS) of the tomato *TGA1* ortholog that resulted in compromised ETI against the virulent pathogen *P. syringae* pathovar (pv.) *tomato* (Ekengren et al., 2003). More recently, Kesarwani et al (2007) demonstrated that a *tgal-1 tga4-1* double mutant of *Arabidopsis* is compromised in basal resistance against virulent *P. syringae*, confirming a role of the TGA1 clade of factors in regulating defense response. The Fobert lab as well as the labs of Dr. Xin Li (University of British Columbia) and Dr. Yuelin Zhang (National Institute of Biological Sciences, Beijing, China) has also observed similar findings with TGA1 clade mutants (unpublished data).

## **2.4 Regulation of SAR Associated Factors Through Post-translational Modification**

In order to perform necessary functions in response to a defined set of conditions, proteins often require PTMs. PTMs are often reversible covalent alterations, which include addition/removal of functional groups, phosphorylation, acetylation, glycosylation, ubiquitination, sumoylation, or oxidoreductive (redox) reactions

(Barford, 2004; Kwon et al., 2006). The consequences of these alterations include, but are not limited to: changes in protein structure, subcellular localization, regulation of activity, stability, and protein-protein or other molecular interactions (Kwon et al., 2006).

#### *2.4.1 Reactive Oxygen Species and Plant Defense Responses*

Changes in the cellular redox balance in response to the generation of ROS are well documented switches for transcriptional control of many cellular processes and have recently been implicated in the regulation of SAR (Grant, 2001; Fobert and Després, 2005). In plants, ROS such as  $\text{H}_2\text{O}_2$  and the superoxide anion ( $\text{O}_2^-$ ) are generated in response to abiotic and biotic stresses (Laloi et al., 2004; Fobert and Després, 2005). Extracellular production of ROS (termed oxidative burst) through plasma membrane-associated NADPH-dependent oxidases is an early feature of ETI (Laloi et al., 2004; Mittler et al., 2004). Common to all ROS cascades are ROS-scavenging enzymes such as catalase, glutathione peroxidase, and peroxiredoxins, which work to detoxify the harmful effects of ROS (Barford, 2004). Also important for coping with oxidative stress are the thiol reductases, thioredoxin (Trx) and glutaredoxin (Grx), which act as thiol reductants to reduce oxidized proteins (Barford, 2004).

Production of  $\text{H}_2\text{O}_2$  is essential for the signal transduction of the SAR response (Alvarez et al., 1998).  $\text{H}_2\text{O}_2$  has been shown to accumulate at the site of infection and is required for the HR (Durrant and Dong; 2004, Mysore and Ryu, 2004). Moreover, secondary oxidative bursts coupled with micro-HRs in non-infected distal tissue and the expression of GST throughout systemic tissues have been observed in plants infected with *P. syringae* (Durrant and Dong; 2004, Mysore and Ryu, 2004). Inhibition of

NADPH-oxidase eliminates the HR in both the infected and distal tissues and thwarts the expression of *GST* and the onset of SAR (Alvarez et al., 1998). Thus, it appears that generation of  $H_2O_2$  in local and distal tissues plays an important role in the SAR response and in the induction of antioxidant genes (Alvarez et al., 1998). Interplay between SA and  $H_2O_2$  in regard to the oxidative burst and induction of SAR remains elusive. However, a study shows that SA is a requirement for the production of HR in response to avirulent pathogens (Thatcher et al., 2005).

There is a dramatic shift in the cellular redox potential in response to exogenous application of the SA analog INA as measured by the levels and ratio of reduced (GSH) to oxidized (GSSG) glutathione (Mou et al., 2003). After an initial decrease of GSH:GSSG after 8 hours, the ratio increases reaching a plateau after 24 hours. Thus, SAR was proposed to be associated with an increase in cellular reduction potential. As subsequently described, this increase in cellular reduction potential is linked to regulation of several proteins associated with SAR (Després et al. 2003; Mou et al 2003).

#### *2.4.2 Redox Regulation of SAR Regulatory Proteins*

Although NPR1 in its uninduced state is present in both the cytoplasm and nucleus (Després et al., 2000), a Green Fluorescent Protein (GFP)-tagged version of this protein was found to exist predominantly in the form of cytosolic oligomers (Mou et al., 2003). In the presence of reducing agents such as dithiothreitol or INA, GFP-tagged NPR1 accumulated as monomers in the nucleus, presumably due to a reduction of intermolecular disulfide bridges between the oligomers (Mou et al., 2003). Site-directed mutagenesis demonstrated that mutation of Cys82 or Cys216 to Ala resulted in

constitutive NPR1 monomerization and the constitutive expression of *PR* genes (Mou et al., 2003). These results indicate that Cys82 and Cys216 may be responsible for forming the intermolecular disulfide bridge that result in NPR1 oligomer formation (Mou et al., 2003).

TGA1 and likely TGA4 have also been demonstrated to be regulated through the redox state of the cell. Through a domain swapping approach a 30 amino acid region of TGA2 was found to be crucial for interaction with NPR1 (Després et al., 2003). Sequence alignment between the seven characterized *Arabidopsis* TGA factors revealed two cysteine residues at positions 260 and 266 in TGA1 that are only present in factors from the TGA1 clade. Site-directed mutagenesis of these Cys to Asn and Ser residues respectively residues thus mimicking the reduced state of this amino acid in TGA1, allowed for its interaction with NPR1 in yeast. Using an *in planta* interaction assay, similar to the yeast two-hybrid system, it was shown that TGA1 could interact with NPR1 following treatment with SA (Després et al., 2003). These results suggested that TGA1 and TGA4 are only capable of interacting with NPR1 when Cys260 and Cys266 are reduced. This hypothesis was further substantiated by measuring the redox status of TGA1/TGA4 cysteines *in vivo*: treatment with SA led to a substantial increase in the pool of reduced TGA1/TGA4 (Després et al., 2003). TGA1 produced *in vitro* and oxidized with the alkylating agent diamide was also shown to have slightly different electrophoretic mobility than reduced TGA1 under non-reducing conditions, consistent with the formation of an intramolecular disulfide bridge involving Cys260 (Després et al., 2003). However, the presence of such a bridge has not been shown experimentally and other options for the state of the oxidized cysteine residues are possible (see section

2.5). Furthermore the redox changes and subsequent oxidation of TGA1 does not affect its ability to bind to its cognate promoter sequence, *as-1*, *in vitro* (Després et al., 2003).

## 2.5 Regulation of Cellular Redox Homeostasis

Protein disulfides come in many forms which give rise to various properties. Inter- and intra- molecular disulfide bonds can mediate protein stability, modulate catalytic activity or mediate a regulatory change (Buchanan and Balmer, 2005). These modifications prevent irreversible damage resulting from the formation of sulfinic (SO<sub>2</sub>H) and cysteinic acids (SO<sub>3</sub>H) that occur when free thiol groups are exposed to an oxidizing environment (Buchanan and Balmer, 2005). These types of stress-induced disulfides play an important role in the regulation of enzymatic proteins and represent a mode of PTMs.

### 2.5.1 Glutathione

The tripeptide glutathione ( $\gamma$ -glutamyl-cysteinyl-glycine) is essential for maintaining the redox status of cytoplasmic protein thiols (Ghezzi, 2005). Its major role is acting as a reactive oxygen scavenger by trapping harmful ROS that may damage proteins with sulfhydryl groups (Ghezzi, 2005). The GSH system is regenerative as it is coupled with glutathione reductase (GR) which reduces GSSG to GSH by accepting protons from NADPH as shown in equation 1 (Ghezzi, 2005). Glutathione moieties can also be added to protein sulfhydryls, in a process called glutathionylation, to protect these SH groups from oxidative damage. A variety of mechanisms for glutathionylation have been identified, including disulfide exchange (eq 2), direct oxidation (eq 3), sulfenic acid intermediate (eq 4 and 5) and formation of S-nitrothiols (eq 6), where PSH

indicates a protein with a free sulfhydryl (Ghezzi, 2005). Some of the aforementioned mixed disulfides make for a more stable bond than others. The hallmark of protein glutathionylation is its reversibility, making redox regulation possible (Ghezzi, 2005). The primary players in reversing protein glutathionylated proteins are the oxidoreductases Gxr and Trx, which mediate the reduction of disulfide bonds, bridges, and GHS-mixed disulfides (Ghezzi, 2005).



### 2.5.2 Thioredoxin and Glutaredoxin

Trxs are small proteins that contain a characteristic Trx fold motif containing 4 alpha helices (Buchanan and Balmer, 2005). In both prokaryotic and eukaryotic cells Trxs are electron donors for peroxidases and possess oxidoreductase activity in response to oxidative stress (Caemel-Harel and Storz, 2000). The *Arabidopsis* genome has the potential to encode 19 different Trxs, grouped into six families with specific cellular locations (Buchanan and Balmer, 2005). The Trx active site is a well conserved WC[G/P]PC motif (Buchanan and Balmer, 2005). Trxs are capable of reducing protein disulfides in all plant cellular compartments through either ferredoxin/thioredoxin reductase or by NADP thioredoxin reductase, where Trx acts as an  $\text{H}^+$  donor to modulate the redox status and accordingly the activity of target proteins (Figure 2.1 a) (Buchanan and Balmer, 2005).

Grxs are small ubiquitous oxidoreductases structurally similar to Trx (Rouhier et al., 2004). Grxs were first identified in *Escherichia coli* mutants lacking Trxs (Holmgren, 1976). Grxs possess activity that is able to compensate for Trx activity as an  $H^+$  donor to ribonucleotide reductase using reducing power supplied by GSH, indicating that Grxs provide an alternative pathway in nucleotide synthesis (Figure 2.1 b) (Holmgren, 1976). Further research concluded that in addition to reduction of ribonucleotide reductase via GSH, initially thought to be the enzyme's primary role, Grxs are potent GSH-disulfide oxidoreductase enzymes (Holmgren, 1979). Grxs were also thought to be able to bind GSH. This was confirmed by NMR studies with a mutant Grx containing a Cys to Ser mutation in the active site containing the consensus sequence CPTC, also known as the signature (Sodano et al., 1991). The mutant monothiol Grx was found to retain some disulfide oxidoreductase activity, while ribonucleotide reductase activity was abolished. The GSH binding site was found to be in the N-terminal portion of the protein (Bushweller et al., 1994), indicating that while the monothiol form of Grx has diminished oxidoreductase activity it is still capable of binding GSH and reducing disulfides. This study was further validated by the identification of monothiol Grxs from both yeast and *E. coli* (*Grx5* and *Grx4*, respectively), which contain the CXXS active site and are considered to be orthologous (Rodriguez-Manzanque et al., 2002; Tamarit et al., 2003; Fenades et al., 2005). Both of these proteins are able to bind GSH at a diminished rate, do not possess any detectable



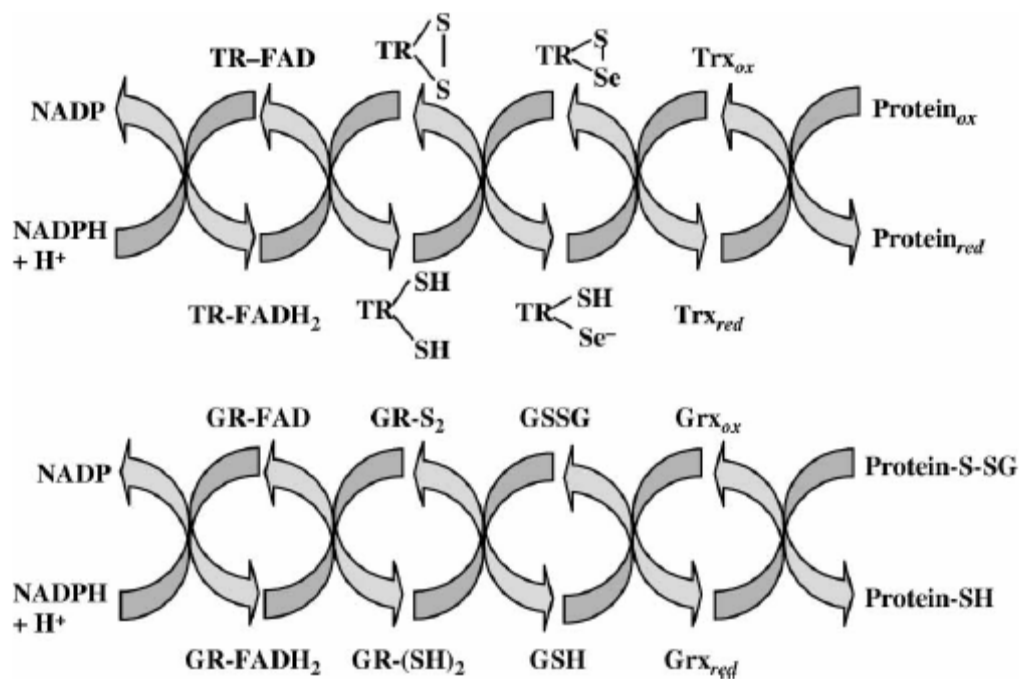


Figure 2.1 Redox Cycle of Thioredoxin (A) and Glutaredoxin (B).

TR, thioredoxin reductase; GR, glutathione reductase. Adapted from Ghezzi et al., 2005.

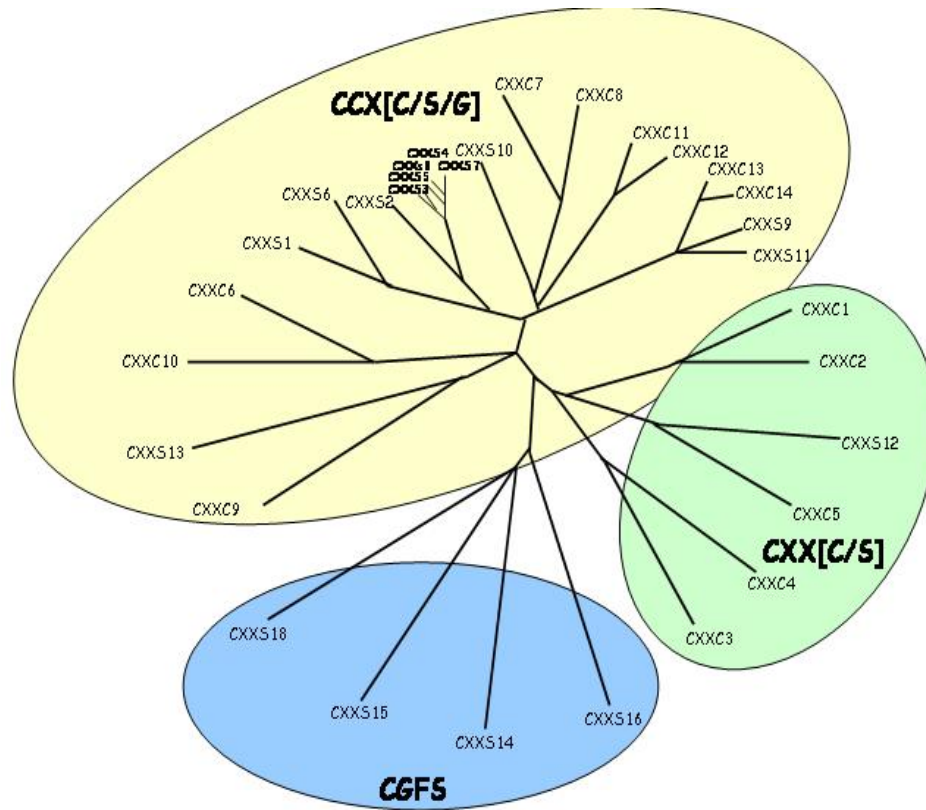


Figure 2.2 Phylogenetic Tree of *Arabidopsis* Glutaredoxin Isoforms.

The three classes are distinguished according to their active site signatures CGFS (PICOT types), CXX[C/S] (Classical types), and CCx[C/S/G] (CC-types). Redrawn from Rouhier et al., 2004

standard disulfide oxidoreductase activity, and may play a role in iron storage (Rodriguez-Manzanque et al., 2002; Tamarit et al., 2003; Fenades et al., 2005).

Grx proteins have also been found to interact with peroxiredoxins, members of the Trx super family; however, their primary function appears to be in mediating the cleavage of mixed disulfides between a specific protein and GSH. A secondary role for the Grx is in electron transport during photosynthesis in plants (Buchanan and Balmer, 2005). There has been little study of Grxs role in plant systems. However, it seems that there is considerable overlap and interplay between the Trx and Grx families (Buchanan and Balmer 2005).

The *Arabidopsis* genome has the potential to encode 31 Grxs that fall into three classes as defined by Rouhier et al., (2004) (Figure 2.2). The “classical” Grxs are well-defined proteins that possess a CXX[C/S] active site signature. The second class belongs to the PICOT-HD (protein kinase C interacting cousin of Trx homology domains) and possesses a CGFS active site. The third class is a large group (encoded by 21 genes) represents CC-type of Grx-like isoforms with CCX[C/S/G] signatures at the putative active site. Members of this group are largely uncharacterized with few reports describing a physiological function (Xing et al., 2005; Xing and Zachgo, 2008). One member of the Grx-like family, *ROXY1*, was identified in a forward genetic screen as loss of its function leads to a plant defective in petal formation (Xing et al., 2005). *ROXY2* was identified by its sequence similarity to *ROXY1* (Xing and Zachgo, 2008). Both *ROXY1* and *ROXY2* contain an active site signature of TCCMC with an N-terminal Cys residue that is essential for function *in planta* (Rouhier et al., 2004; Xing et al., 2005; Xing and Zachgo, 2008). Studies revealed that knock-out mutants of both

genes lead to sterility and lack of pollen production (Xing and Zachgo, 2008). Mutation of a conserved Gly residue (Gly-110 to Ala) in ROXY1, required for glutathione binding, was unable to restore wild type flower morphology in the *roxy1-5* mutant background (Xing and Zachgo, 2008). Furthermore, molecular modeling, based on the classical Grx CXXC1, revealed that ROXY1 and ROXY2 likely possess a typical Trx fold structure where both the conserved Gly 110 and the N-terminal Cys are exposed on the surface of the predicted protein (Rouhier et al., 2004; Xing and Zachgo, 2008). These results suggest that the Grx-like proteins, ROXY1 and ROXY2, are likely able to bind and interact with glutathione and other molecules.

A recent study reported that TGA2 physically interacts with the Grx-like protein CXXC9 in yeast-2 hybrid assays (Nadamukong et al., 2007). These results agree with previous observations in the Fobert lab and can be further extended to include TGA1, TGA3, a member of the TGA3 clade, as well as TGA2, which were found to interact with all of the Grx-like proteins tested (Fobert et al., unpublished). Additionally, microarray data indicated that transcript levels of several Grx-like genes increased in a *tgal-1 tga4-1* double, loss-of-function mutant, indicating that TGA1/TGA4 may act as negative regulators of Grx-like gene expression (Fobert et al., unpublished). Furthermore, some of the Grx-like genes are differentially expressed in the *npr1* mutant (Fobert et al., unpublished). These results, combined with the biochemical characterization of ROXY1 and ROXY2 suggest that the Grx-like proteins may be involved in the regulation of TGA1 properties through redox PTM, and that TGA1 and its interacting partner NPR1 may mediate the expression of the same Grx-like genes that encode these enzymes.

## 2.6 Redox Regulation of Regulatory Proteins in Non-Plant Systems

It is not yet known what mechanism is responsible for redox regulation of NPR1, TGA1 and TGA4, or the potential role of oxidoreductases in this process; however, there are several well-studied systems from *E. coli*, yeast, and mammals that may lend clues as to the possible mechanisms at play.

### 2.6.1 OxyR

OxyR is an *E. coli* transcription factor which is sensitive to the internal redox status of the cell and is activated by oxidation that causes rapid formation of a reversible disulfide bond in the presence of H<sub>2</sub>O<sub>2</sub> (Zheng et al., 1998; Åslund et al., 1999; Toledano et al., 2004; Barford, 2005). Oxidation occurs on Cys199 and Cys208 and results in a conformational change in its regulatory domain leading to reassortment of oligomeric protein interactions, activation of OxyR, derepression of the OxyR regulon, and expression of genes encoding enzymes that catalyze the formation of antioxidants i.e. antioxidant genes (Zheng et al., 1998; Choi et al., 2001; Barford, 2005). The disulfide bonds of active OxyR are likely to be subsequently reduced by the glutaredoxin Grx1 as the cellular GSH:GSSG ratio increases (Zheng et al., 1998). The *Grx1* gene is regulated by OxyR suggesting an autoregulatory loop (Zheng et al., 1998; Barford, 2005). Interestingly, OxyR is able to respond to other types of oxidative stress in addition to H<sub>2</sub>O<sub>2</sub>. It was revealed that oxidation with various compounds, such as nitrothiols and thiol oxidants, was able to alter the structure and DNA affinity of OxyR, giving rise to a graded response to oxidative stress and the formulation of the hypothetical redox code (Kim et al., 2002; Toledano et al., 2004).

### 2.6.2 *Yap1-Orp1*

In budding yeast the Yap1 bZip transcription factor controls a regulon encoding antioxidant proteins (Barford, 2005). Unlike OxyR, which appears to sense redox conditions directly, activation of Yap1 is dependent on a redox sensor called Orp1 which functions as an on/off switch in response to H<sub>2</sub>O<sub>2</sub> (Toledano et al., 2004; Barford, 2005). Orp1 is a member of the peroxiredoxin family and is oxidized by H<sub>2</sub>O<sub>2</sub>. Initially, an intermolecular disulfide bond forms between Cys598 of Yap1 and Cys36 of Orp1. This bond is then transformed to an intramolecular disulfide bridge between Cys303 to Cys598 of Yap1, resulting in a substantial conformational change, that unveils a nuclear localization signal and leads to subsequent nuclear localization, and transcription of target antioxidant genes (Barford, 2005).

### 2.6.3 *NF-κB/IκB*

NF-κB is a mammalian transcription factor that is activated in response to immune stimulation from tumor necrosis factor-α, interleukin-1, and LPS (Kabe et al., 2005). In its inactive form NF-κB is held captive in the cytoplasm by its inhibitory partner IκB, which has some sequence similarity to NPR1 (Ryals et al., 1997; Kabe et al., 2005). IκB is a repressor of NF-κB in the cytoplasm. Phosphorylation of IκB at Ser/Thr residues results in its ubiquitination and subsequent proteolytic cleavage, allowing NF-κB to be released and relocated to the nucleus where it activates expression of target genes (Kabe et al., 2005). NF-κB activity is regulated at multiple steps by redox conditions. First, the mitogen activated protein (MAP) kinase responsible for phosphorylating IκB is activated in response to ROS (Kabe et al., 2005). Second, nuclear NF-κB must undergo reduction of Cys62 to adopt its active state (Kabe

et al., 2005). Cys62 is present in the DNA-binding domain of NF- $\kappa$ B, and its oxidation can decrease NF- $\kappa$ B's DNA binding ability while its reduction enhances DNA binding (Kabe et al., 2005). Trx, which can translocate to the nucleus following immune stimulation, has been implicated in the reduction of nuclear NF- $\kappa$ B's Cys62. Of note, prior to stimulation, Trx acts as a negative regulator of cytoplasmic NF- $\kappa$ B by inhibiting parts of the MAP kinase pathway (Kabe et al., 2005). Nuclear NF- $\kappa$ B has also been shown to be negatively regulated through oxidative nitrosylation of Cys62 (Kabe et al., 2005). Interestingly, NF- $\kappa$ B is a positive regulator of nitric oxide (NO) synthase (Kabe et al., 2005).

#### *2.6.4 ASK1 Kinase*

The mammalian ASK1 is a member of the MAP kinase family that is involved in apoptotic signaling, and can be activated by various stress conditions including ROS (Saitoh et al., 1998). In the absence of ROS, and hence more reducing conditions, Trx binds to the N-terminal region of ASK1 and inactivates it. However, the presence of cytotoxic stresses induces the oxidation of Trx, inhibiting its ability to bind to ASK1 and thus enables ASK1 to become an active kinase (Saitoh et al., 1998). Thus, ASK1 can be thought to sense ROS through its interaction with Trx. Note that unlike the other examples provided, ASK1 cysteines are not reduced by Trx (Saitoh et al., 1998).

#### *2.6.5 AP-1*

AP-1 consists of two proto-oncogenes, c-Fos and c-Jun, which can form hetero- or homo-dimers (c-Jun only), and are members of the family of bZip transcription factors in mammalian systems (Sen and Packer, 1996). Of note, TGA1, also a member of the bZip superfamily (section 2.2.3), has been proposed to be related to c-Fos and c-

Jun (Després et al., 2003). c-Fos and c-Jun are known to interact with a DNA regulatory element called the activator protein-1(AP-1) binding site (Sen and Packer, 1996). In order for AP-1 to bind to its DNA regulatory element it requires the reduction of conserved cysteine residues in the DNA binding domains of the hetero- or homodimers, while oxidation of this cysteine residue inhibits its DNA binding capability (Abate et al., 1990). Reduction of AP-1 is mediated by a DNA repair enzyme, Ref-1, that physically interacts with a Trx (Hirota et al., 1997). Ref-1 localizes to the nucleus several hours after exposure to ROS where it interacts with AP-1. The physical interaction of Ref-1 with Trx suggests that there may be additional PTMs required for this localization event to occur (Ramana et al., 1998).

## **2.7 Research Goals**

The working hypothesis is that the interaction between Grx-like proteins and TGA1/TGA4 is relevant to the redox regulation of these transcription factors. Accordingly, I propose to address the following key questions:

- (i) Are the Grx-like genes expressed in a manner consistent with a role in response to pathogens? Do transcript levels of these genes respond to SA or pathogen infection? Are the genes regulated by TGA1 or NPR1?
- (ii) Does altering the expression of the Grx-like proteins CXXC9 and CXXS13 result in altered tolerance to oxidative stress, pathogens, or changes in defense gene expression?
- (iii) Do the Grx-like proteins possess oxidoreductase activity? Will they reduce a well-known Grx substrate?



By addressing these questions this research begin to elucidate the role of the Grx-like proteins in the plant defense response.

## **Chapter 3: Materials and Methods**

### **3.1 Chemicals**

All chemicals, reagents, and enzymes were purchased from Sigma-Aldrich (Oakville, ON) or Calbiochem (La Jolla, California USA) unless otherwise stated. All oligonucleotides were synthesized by the DNA Technology Service at the National Research Council - Plant Biotechnology Institute (Saskatoon, Saskatchewan).

### **3.2 Cloning Glutaredoxin-Like Genes**

Unless otherwise noted, all standard molecular cloning protocols were performed, and reagents prepared, as described in Sambrook and Russell (2001). The coding regions for genes of interest were amplified using the polymerase chain reaction (PCR) using oligos described in Table 3.1. The 5' and 3' oligonucleotides were designed to introduce a NotI and a SalI restriction site, respectively. The 3' oligonucleotides contain no stop codon to allow read-through into the C-terminal 6X His tag located in the cloning vector. PCR products were ligated directly into the PCR<sup>®</sup>2.1-TOPO vector and transformed in *Escherichia coli* DH12S cells as recommended by the manufacturer (Invitrogen, Burlington, Ontario). Coding regions were subsequently subcloned into a modified version of the protein expression vector pET41a (Novagen, Mississauga, Ontario) using the restriction sites NotI and SalI. The integrity of gene inserts was confirmed by sequence analysis.

Table 3.1 Oligonucleotides for Cloning

1. Nomenclature as in Rouhier et al. 2004

2. *Arabidopsis* Gene Identifier (AGI) numbers are as indicated by The *Arabidopsis* Information Resource website <http://www.arabidopsis.org/>

Name <sup>1</sup>	Oligo Sequence (5' - 3')	Length	Protein Entry Code <sup>2</sup>
<b>CXXC1-5'</b>	ATGGCGGCCGCAGTTGAGAAGAGTTATC	28	At4g15660
<b>CXXC1-3'</b>	ATGGTCGACGATGGAGAAGATACAAAAGATGA	32	
<b>CXXC7-5'</b>	ATGGCGGCCGCAGCCAGAGAGCGCC	25	At3g02000
<b>CXXC7-3'</b>	TAGGTCGACGATGCAATACCAGACAGAATC	30	
<b>CXXC8-5'</b>	ATGGCGGCCGCAGCCAAAGAGCGCCAGC	28	At5g14070
<b>CXXC8-3'</b>	ATGGTCGACGATGCAATACAAAACAGAAAC	30	
<b>CXXC9-5'</b>	CAAGGGTCGACAATGCAAGGAACGATTTC	29	At1g28480
<b>CXXC9-3'</b>	ATGGCGGCCGCAACCACAGAGCCCCAAC	28	
<b>CXXS13-5'</b>	ATGGCGGCCGCAGCCATAAAGCCCCAG	27	At1g03850
<b>CXXS13-3'</b>	CAAGGGTCGACTATGCAAAAAGCAATTCGAC	31	

### 3.3 Expression and Purification of Recombinant Glutaredoxin-Like Proteins

pET41a derivatives containing the coding regions for *Arabidopsis* glutaredoxin genes were individually transformed into *E. coli* BL21 (DE3) pLysS cells (Novagen) for production of recombinant proteins. Bacterial cultures were grown to an O.D. of 0.4-0.6 ( $A_{600}$ ) at 37 °C and 1 mM isopropyl  $\beta$ -D-1-thiogalactopyranoside (IPTG) was added to induce recombinant proteins production. Cultures were grown for ~ 4 h at 37 °C, centrifuged, and pellets were stored at -20 °C.

Lysis of bacterial cells was performed as per the method of Fangioni and Neel (1993) with the following modifications. Cells were incubated on ice with 1 mM phenylmethanesulphonylfluoride (PMSF), 5 mM dithiothreitol (DTT), 0.1 mg/mL lysozyme, and 1% N-laurylsarcosine (sarkosyl) in STE (10 mM Tris, 150 mM NaCl, 1 mM Disodium ethylenediaminetetraacetate [EDTA]) buffer for 30 min. Cells were sonicated (XL, Heat Systems, Farmingdale, NY) on medium power for 3-5 min or until lysates turned transparent. The lysate was centrifuged for 15 min and supernatant was transferred to 6-8000 MWCO dialysis tubing (Spectra/Por®, Rancho Dominguez, CA, USA) and dialyzed against Native purification buffer (see below) with 1% Triton X-100 for 24 h with three buffer changes at 4° C.

Purification of recombinant proteins under non-denaturing (native) conditions was performed using the Ni-NTA purification system (Invitrogen; Burlington, Ontario). All buffers, reagents, and procedures were performed as indicated by the manufacturer with the following modifications. Native binding buffer was prepared with 20 mM imidazole to equilibrate the column. Approximately 8 mL of protein lysate was added to the column and mixed for 30-60 min at 4 °C. The unbound protein was washed off the

column 3 times using 8 mL native wash buffer with 30 mM imidazole. A fourth wash was performed with native wash buffer with 30 mM imidazole, pH 5.0. The bound protein was eluted off the column with 10 mL native elution buffer with 250 mM imidazole and collected in several different fractions of 1 mL each.

### **3.3.1 Assessment of Protein Purity**

The purity of the recombinant proteins was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by staining with Coomassie Brilliant Blue R 250.

### **3.3.2 $\beta$ -Hydroxyethyl Assay**

The  $\beta$ -hydroxyethyl (HED) assay was performed as per Holmgren and Åslund (1995) with the following modifications. In a final volume of 250  $\mu$ L were mixed 1 mM reduced glutathione (GSH), 0.7 mM  $\beta$ -hydroxy ethyl disulfide (HED), 0.4 mM nicotinamide adenine dinucleotide phosphate (NADPH), 2 mM EDTA, 0.1 mg/mL bovine serum albumin (BSA), 6.4  $\mu$ g/mL glutathione reductase from baker's yeast in 0.1 M Tris-Cl pH 8.0. The reaction mixture was incubated at room temperature for 2 min to allow the HED substrate and the GSH to form a mixed disulfide. The total amount of glutaredoxin (Grx) added to the reaction mixture was 1  $\mu$ g. The reaction was monitored as a decrease in absorbance of NADPH to NADP<sup>+</sup> at 340 nm in a SpectroMax micro-plate reader (Union City, California, USA) over a 5 min period at room temperature.

### 3.4 Plant Material

Unless otherwise noted, all *Arabidopsis thaliana* plants, wild-type or mutants, were in the ecotype Columbia genetic background. The *tga1-1* and *tga4-1* mutants (Salk\_028212 and Salk\_127923, respectively), were generated by the Salk Institute Genome Analysis Laboratory (SIGnAL) project (Alonso et al., 2003) and obtained from the *Arabidopsis* Biological Resource Center (ABRC; Columbus, Ohio, USA). The *tga4/tga1* and *tga1/tga4* double mutants were derived from reciprocal crosses of the *tga1-1* and *tga4-1* mutants in the Fobert lab. The *npr1-3* mutant was obtained from ABRC. Two CXXC9 overexpression lines had been previously generated in the Fobert lab by transgenic stable expression of the CXXC9 gene under the influence of Cauliflower mosaic virus 35 S promoter (CaMV35:CXXC9) and confirmed by northern blot analysis (Tracy Wignes, unpublished results). The CXXC9 overexpression lines were selfed to the T<sub>2</sub> generation and lines that consistently expressed the transgene were analyzed in this study. T-DNA insertion lines in CXXC9 (Salk\_074219; T-DNA inserted just after the 5'UTR) and CXXS13 (Salk\_074219; T-DNA inserted in first exon) and were obtained from ABRC.

#### 3.4.1 Growth Conditions and Treatments

In the initial experiments (qRT-PCR Repetition 1), plants were sown on soil (Sunshine No.4 mix; Seba Beach, Alberta), stratified at 4 °C for 2-3 days and placed in a growth cabinet with a photoperiod of 10 h light at 100 µE and fertilized with 20-20-20 (Plant-Prod®; Crossfield, Alberta).

*Pseudomonas syringae* pathovar *tomato* DC3000 (virulent strain) (Kus et al. 2002) was grown in 2YT medium at 30 °C for sixteen h, collected by centrifugation then resuspended in 10 mM MgCl<sub>2</sub> and diluted to 1 X 10<sup>6</sup> colony forming units (cfu) mL<sup>-1</sup>. Six-week-old plants were either inoculated with *P. syringae*, sprayed with 0.5 mM sodium salt of salicylic acid (SA) or H<sub>2</sub>O until imminent runoff. Tissue was collected for *P. syringae* inoculated plants 24 h and 48 h after treatment. Tissue for SA treated plants was collected 4 h and 8 h after treatment. Tissue for H<sub>2</sub>O treated plants was collected 8 h after treatment.

In subsequent experiments (repetitions 2-6), seeds were surface sterilized in a 10% bleach solution, washed extensively in sterile water, stratified for 2-3 days at 4°C, plated on Murashige and Skoog medium (MS) solidified with 0.62 % phytagel (Sigma) and placed in a growth cabinet with a photoperiod of 10 h light at 150 µE. Seedlings were transplanted to soil (Sunshine No.4 mix) 8 days after germination. Three weeks after germination, plants were treated with 0.5 M SA or 20 µM methyl jasmonic acid (MeJA) dissolved in 0.2 % ethanol; control plants were sprayed with either H<sub>2</sub>O or 0.2 % ethanol, respectively. Tissue for SA treated material was collected 1 h and 8 h after treatment. Tissue for MeJA treated material was collected at 4 h and 8 h after treatment. Tissue for H<sub>2</sub>O treated plants was collected 8 h after treatment.

Seeds for repetition 7 were sterilized and plated on Murashige and Skoog (MS) solid medium and placed in a growth cabinet with 20 °C, 100 µE, 16 h light /8 h dark cycle. Plantlets were transferred to soil (Sunshine No.4 mix) ~8 days after germination. JA treatments and tissue was collected as indicated above for repetitions 2-6.

### 3.4.2 Paraquat Assay

Sterile, stratified seeds were plated on MS solidified medium with paraquat at 50 or 100 nM, or control plates (no paraquat). Plates were placed in vertical racks and incubated in a growth chamber under the following conditions: 150  $\mu$ E and, 10 h day. Plantlets were removed from the plate and measured with a ruler 12-14 days after incubation.

### 3.4.3 Disease Test

*Pseudomonas syringae* DC3000 (pVSP61) and *P. syringae* DC3000 (pVSP61 + *avrRpt2*) (Kus et al., 2002) were grown in Kings B medium (Sambrook and Russell, 2001) with 50mg/L kanamycin and 100 mg/L rifampicin or 50 mg/L kanamycin, respectively. The bacterial cultures were grown for ~16 h at 28 °C or until they reached an O.D. of 0.3-0.6 ( $A_{600}$ ). Cells were collected by centrifugation and resuspended in 10 mM  $MgCl_2$  for a final concentration of  $1 \times 10^6$  cfu/mL for the avirulent and  $1 \times 10^5$  cfu/mL for virulent strains.

Plants were grown as previously described in section 3.4.1. Sixteen days after germination two leaves on each plant were inoculated, using a 1 mL plastic syringe, with either 10 mM  $MgCl_2$  or *P. syringae* DC3000 *avrRpt2*. Three to four leaves on each plant were subsequently challenged with *P. syringae* DC3000 (virulent) 3 days after the initial pre-treatment. Three days later 8 leaf disks (4 mm in diameter) were harvested from the challenge leaves. Leaf samples were ground in 500  $\mu$ L  $MgCl_2$ , serially diluted to 1/5000, and plated on King's B plates. The plates were incubated at room temperature for 2-4 days before counting colonies.



### 3.5 Quantitative Polymerase Chain Reaction Assays

All tissues were collected at approximately 3:30 – 4:00 pm to minimize circadian influence. Samples contained 100 mg of tissue flash frozen in liquid nitrogen and stored at -80 °C.

Total RNA was extracted using the RNeasy® mini kit (Qiagen, Mississauga, Ontario) as per the manufacturer's instructions, including the on-column DNase treatment.

RNA was quantified using a NanoDrop® ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies; Wilmington, Delaware, USA). An aliquot containing 2 µg of RNA was measured and brought up to a total volume of 8 µL in H<sub>2</sub>O. Prior to cDNA synthesis, a second DNase treatment was performed. In a total volume of 10 µL 1 U of DNase I (Invitrogen), 2 µg RNA and 10 X DNase I reaction buffer (supplied with DNase I) were incubated for 15 min at room temperature. The DNase I was deactivated by addition of 2.5 mM EDTA and incubation at 60 °C for 10 min. First-strand cDNA synthesis was performed in a total volume of 20 µL with 2.5 pmol Oligo (dT)<sub>17</sub>VN (Table 1.2), 0.1 mM deoxynucleotide triphosphate (dNTP) (Invitrogen), 0.4 U RNasin (Fisher, Ottawa, Ontario), 10 mM DTT, 2.5 U SuperScript II reverse transcriptase (Invitrogen), 5 X first-strand synthesis buffer and H<sub>2</sub>O in the DNase treated RNA. The reaction was incubated in a thermocycler under the following conditions: 42 °C for 50 min for first-strand synthesis followed by 70 °C for 15 min. The newly synthesized cDNA was then diluted 1/200 to reflect a concentration of 10 ng/µL with respect to the initial total RNA aliquot.

qRT-PCR reactions were performed in a total volume of 12.5  $\mu$ L containing 0.5 pmol oligonucleotides (see Table 3.1.3 for oligo information), 5 ng cDNA, 1 X SYBR Green® mix (Quantitech; Qiagen), and H<sub>2</sub>O. All amplifications were conducted with a Mx3000P spectrofluorometric thermal cycler (Stratagene) using a two temperature cycling regime as follows: 15 min 95 °C, 10 sec 95 °C denaturing, 2 min 66 °C annealing/extension for 40 cycles. The fluorescence data collected at the end of each qRT-PCR cycle was subject to linear regression (Rutledge and Stewart, 2008). The absolute numbers of initial DNA at the start of the reaction are then calculated by taking into account the size of the DNA amplicon, the weight of starting DNA, a standard of fluorescence calculated from lambda phage, and the calculated efficiency of the oligos used in the amplifications (Rutledge and Stewart, 2008).

### **3.6 Statistical Treatment of Oxidative Stress and Disease Tests**

In order to determine whether the differences observed in the paraquat assays are statistically significant a 1-way analysis of variance (ANOVA) was used. The results of the 1-way ANOVA were generated by the statistical analysis package in Microsoft Excel. The *p* value was set at  $\alpha = 0.05$ . The Tukey's test was used to validate the results of the 1-way ANOVA, calculations were derived using an online calculator (<http://web.mst.edu/~psyworld/tukeycalculator.htm>).

In order to determine whether the differences observed in the disease test are statistically significant a 2-way ANOVA was used. The results of the 2-way ANOVA were generated by the statistical analysis package in Microsoft Excel. The *p* value was set at  $\alpha = 0.05$ . A student's T-test was used to validate the results of the 2-way ANOVA

and was also generated by the statistical analysis package in Microsoft Excel. The  $p$  value was also set to  $\alpha = 0.05$ .

Table 3.2 Oligo for cDNA Synthesis

Name	Oligo Sequence	Length	Target
DT17VN	TTTTTTTTTTTTTTTTTT(A/G/C)(A/C/G/T)	19	PolyA tail of mRNA

Table 3.3 Oligos for qRT-PCR

All primers were chosen to be specific for their target gene as determined by sequence alignment. The free software Primer 3 <http://frodo.wi.mit.edu/> was used to design the primers

Name	Oligo Sequence	Length	Amplicon size	Target
CXXC9-5'	TTGGAACCTAACTCACCGGAGATATGAGTAG	30	148	At1g28480
CXXC9-3'	GAAGATGAAGTTTGTAGTGAGTTGGAGAAT	30		
CXXC8-5'	CAAGTGCTCACGCTGAATATAACCAC	26	119	At5g14070
CXXC8-3'	TCCTACAACAACAACAGTAAGGTGATGAAC	30		
CXXS8-5'	GAGAAGATACAAAAGATGATCTCCGAGAAG	30	166	At4g15660
CXXS8-3'	CCAATGCATACTCTATCTCTTTTCTCTGT	30		
CXXS13-5'	ACCAAAGCCAACATCTATAAGCAATAAGAG	30	178	At1g03850
CXXS13-3'	GTAGTTGTTGTTGTCTTCTTCACCAATCTC	30		
PR1-5'	GCTCTTGTAGGTGCTCTTGTCTTCC	26	173	At2g14610
PR1-3'	AGTCTGCAGTTGCCTCTTAGTTGTTTC	26		
UBQ5-5'	AGCTTACAAAATTCCCAAATAGAAATGCAG	30	117	At3g62250
UBQ5-3'	ACCTACGTTTACCAGAAAGAAGGAGTTGAA	30		
PDF1.2-5'	CTTATCTTCGCTGCTCTTGTTCTCTTTG	29	134	At5g44420
PDF1.2-3'	CACTGATTCTTGCATGCATTACTGTTTC	28		

## Chapter 4: Results

### 4.1 Expression of *PR-1* in Response to Salicylic Acid and Pathogen

In microarray experiments using *Arabidopsis* rosette leaves, transcripts for the gene encoding Grx-like protein CXXS8 was found to be upregulated in the *tga1-1 tga4-1* double mutant 8 h after treatment with SA. Furthermore, CXXC9, CXXC8, and CXXS13 were found to interact with several members of the TGA family of transcription factors, including TGA1 (Fobert et al., unpublished). These Grx-like genes were chosen to further analyze their role in the defense pathways based on these findings. Analysis of expression of mRNA transcript in wild type *Arabidopsis* following treatment with SA or challenge with pathogens will give suggestions as to whether genes encoding the Grx-like proteins are expressed in a manner that is consistent with a role in defense responses. Quantitative reverse-transcriptase-PCR (qRT-PCR) is a method that measures absolute amounts of mRNA transcripts in a sample through PCR of cDNA synthesized from mRNA, thus allowing comparison of selected genes and their expression profiles. Due to biological variation it was necessary to repeat the experiments several times in order to have confidence in the results.

*Pathogenesis Related (PR)* genes are highly induced after pathogen attack or exogenous application of SA (Durrant and Dong, 2004). Thus *PR* genes serve as useful markers for pathogen infection. Measurement of *PR-1* transcript levels was initially done to ensure that plants were mounting a defense response under the experimental conditions prior to measuring the expression of Grx genes

Table 4.1 Fold Changes of Transcript Levels in Wild Type Leaves

A cut off value of 2 fold increased or decreased transcript level was set arbitrarily to indicate changes in transcript levels. Green highlight indicates increase in transcript levels, over wild type leaves, after treatment with SA or *P. syringae* while red indicated a decrease in transcript levels. No highlight indicates no change in transcript levels, from wild type leaves, after treatment with SA or *P. syringae*. Grey highlight indicated no data for a replicate set. R1, R2, R3, and R4 = replicate 1, replicate 2, ect. h = hour.

A cut off value of 2 fold increased or decreased transcript levels were set arbitrarily to indicate changes in transcript levels. Green highlight indicates increase in transcript levels, over wild type leaves, after treatment with SA or *P. syringae*. No highlight indicates no change in transcript levels, from wild type leaves, after treatment with SA or *P. syringae*. Grey highlight indicated no data for a replicate set. R1, R2, R3, and R4 = replicate 1, replicate 2, ect. h = hour.

	R1		R2		R3		R4	
SA	Fold change 4 h	Fold change 8 h	Fold change 1 h	Fold change 8 h	Fold change 4 h	Fold change 8 h	Fold change 4 h	Fold change 8 h
PR1	65.22	25.95	0.63	146.22	1792.41	11688.32	207.46	1962.40
CXXC9	8.54	9.48	3.64	22.80	95.60	77.09	33.91	21.83
CXXS13	4.23	2.83	6.26	22.46	143.64	791.61	9.03	6.98
CXXC8	0.00	0.00	11.12	1.53	2.22	9.79	-10.68	-8.44
CXXS8	-3.53	-1.20	1.05	1.10	1.01	-2.95	-12.21	-3.07
<i>P. syringae</i>	Fold change 24 h	Fold change 48 h			Fold change 24 h	Fold change 48 h		
PR1	12940.98	25653.77			16474.49	105957.56		
CXXC9	34.33	85.57			466.37	280.00		
CXXS13	43.85	27.60			554.06	275.76		
CXXC8	0.00	0.00			5.42	3.42		
CXXS8	-1.37	-14.36			-1.28	-10.68		

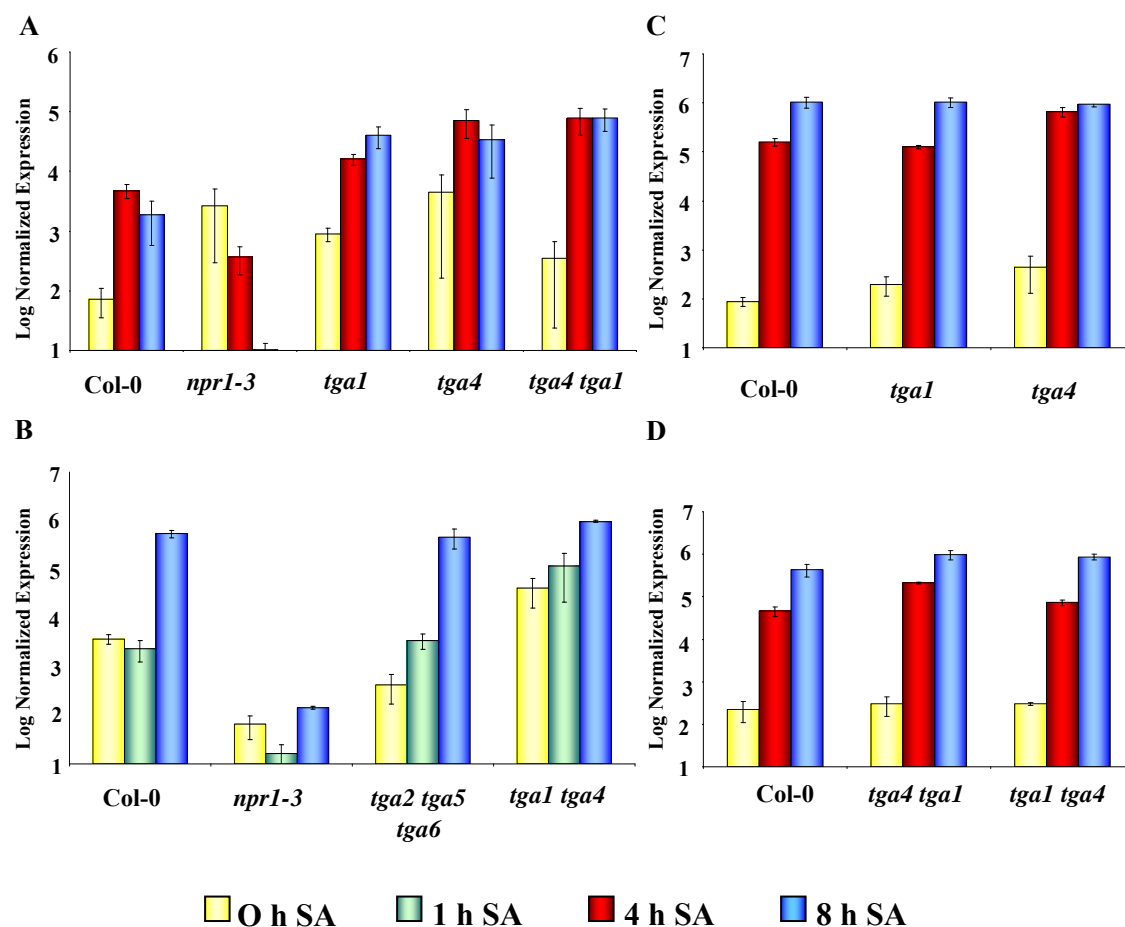


Figure 4.1 Analysis of *PR-1* Expression Following Treatment with Salicylic Acid by qRT-PCR.

Changes in mRNA levels were assayed over a time course after SA treatment. Each bar represents 3 biological replicates, with 4 qRT-PCR technical replicates (A) or 2 technical replicates (B, C, D) represented as the average of biological replicates. The numbers of RNA transcripts normalized against *UBIQUITIN5*. Error bars represent standard error. A, B, C, and D refer to R1, R2, R3, and R4 respectively.

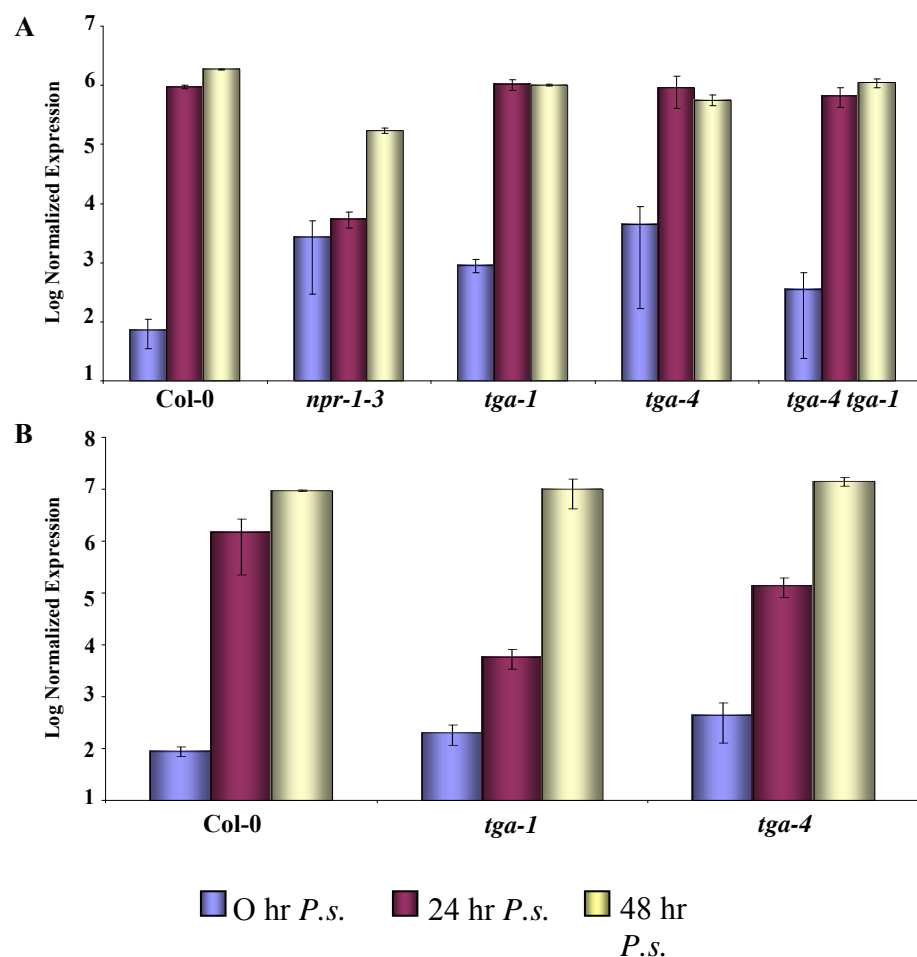


Figure 4.2 Analysis of *PR-1* Expression Following Treatment with *Pseudomonas syringae* by qRT-PCR.

Changes in mRNA levels were assayed over a time course after *P. syringae* treatment. Each bar represents 3 biological replicates, with 4 qRT-PCR technical replicates (A) or 2 technical replicates (B) represented as the average of biological replicates. The numbers of RNA transcripts were normalized against *UBIQUITIN5*. Error bars represent standard error. A and B refer to R1 and R3 respectively.



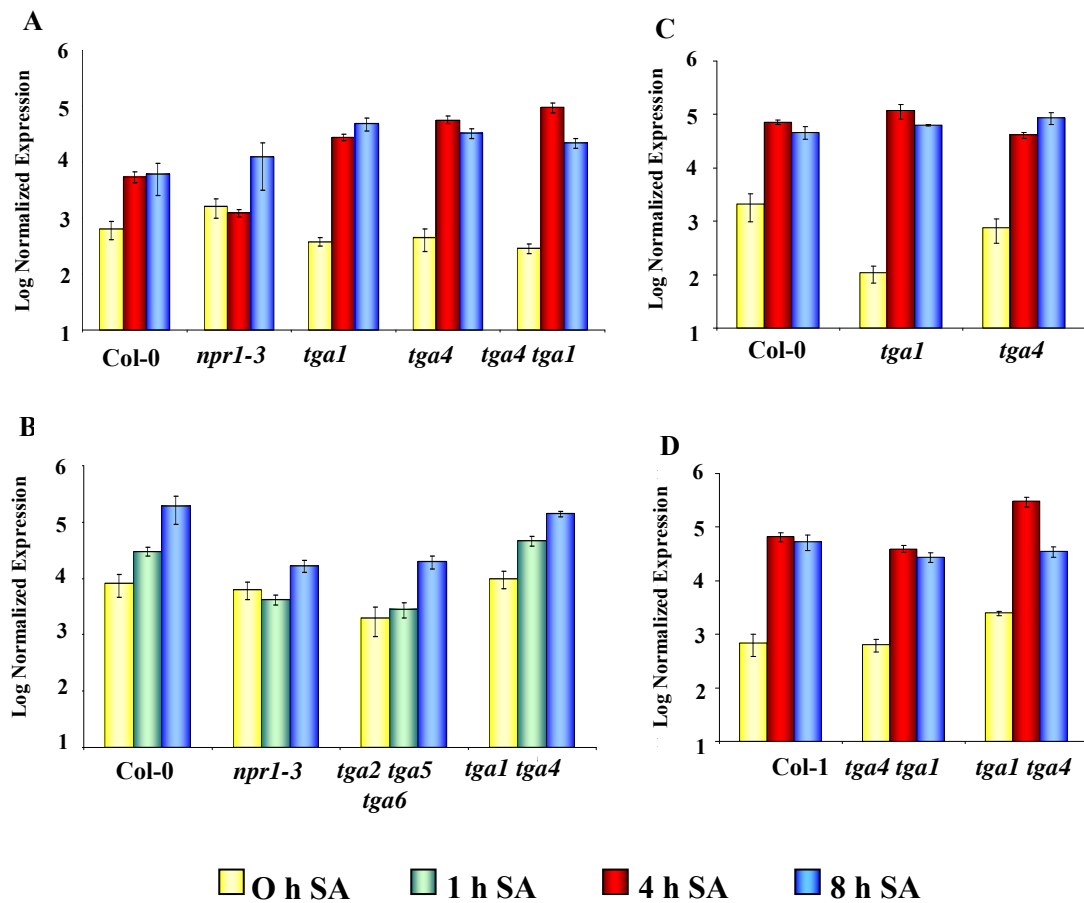


Figure 4.3 Analysis of *CXXC9* Expression Following Treatment with Salicylic Acid by qRT-PCR.

Changes in mRNA levels were assayed over a time course after SA treatment. Each bar represents 3 biological replicates, with 4 qRT-PCR technical replicates (A) or 2 technical replicates (B, C, D) represented as the average of biological replicates. The numbers of RNA transcripts were normalized against *UBIQUITIN5*. Error bars represent standard error. A, B, C, and D refer to R1, R2, R3, and R4 respectively.

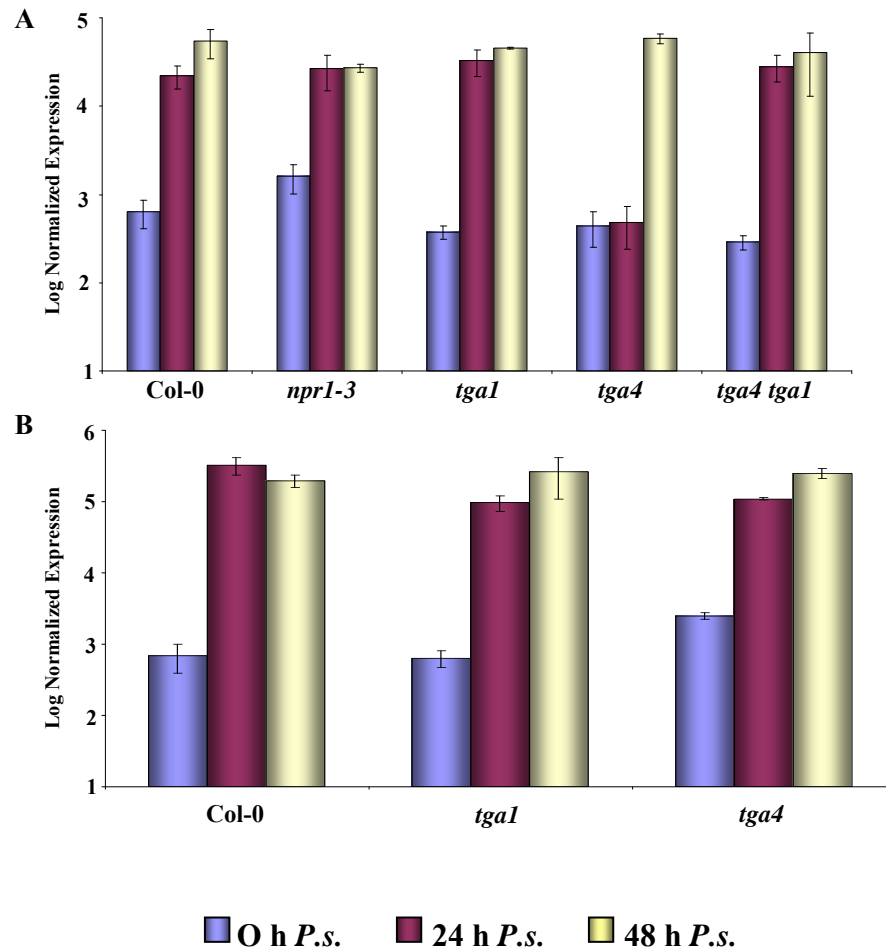


Figure 4.4 Analysis of *CXXC9* Expression Following Treatment with *Pseudomonas syringae* by qRT-PCR.

Changes in mRNA levels were assayed over a time course after *P. syringae* treatment. Each bar represents 3 biological replicates, with 4 qRT-PCR technical replicates (A) or 2 technical replicates (B) represented as the average of biological replicates. The numbers of RNA transcripts were normalized against *UBIQUITIN5*. Error bars represent standard error. A and B refer to R1 and R3 respectively.

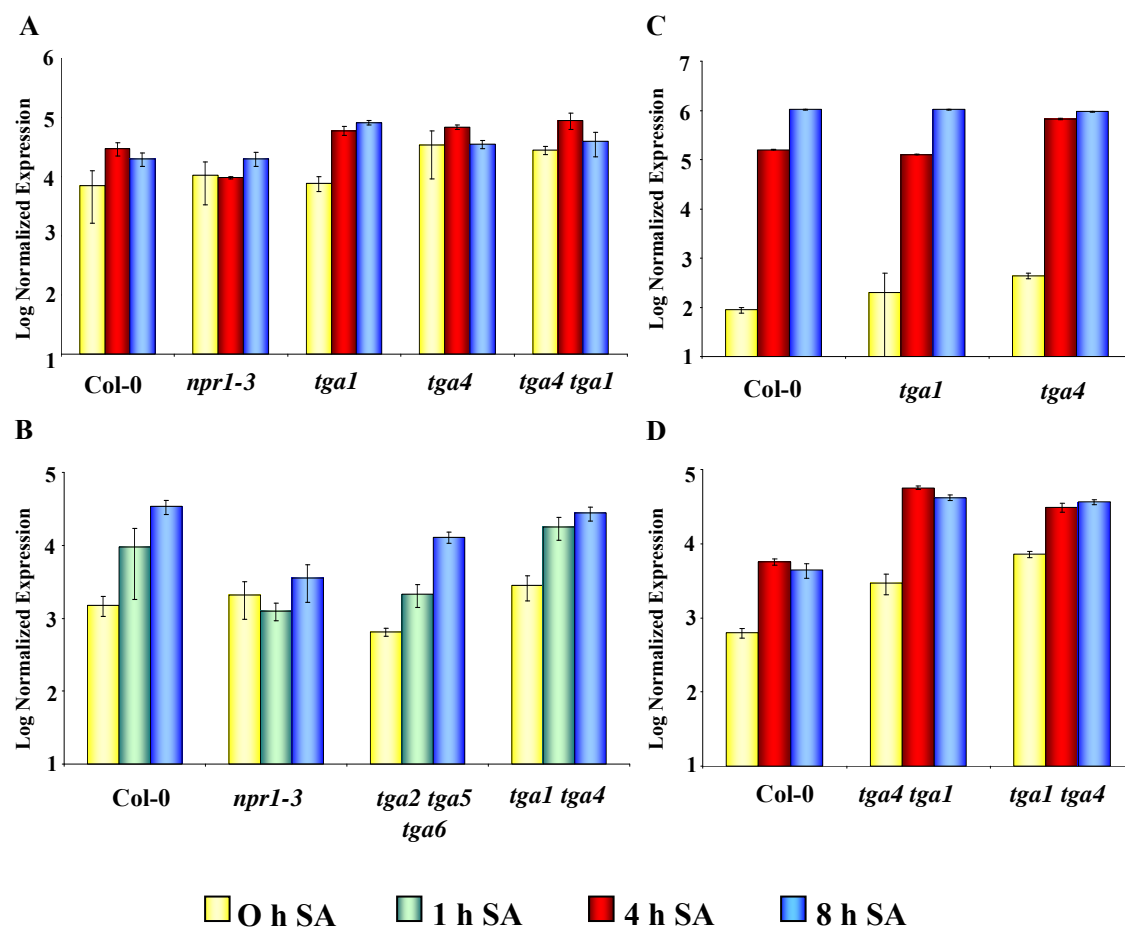


Figure 4.5 Analysis of *CXXS13* Expression Following Treatment with Salicylic Acid by qRT-PCR.

Changes in mRNA levels were assayed over a time course after SA treatment. Each bar represents 3 biological replicates, with 4 qRT-PCR technical replicates (A) or 2 technical replicates (B, C, D) represented as the average of biological replicates. The numbers of RNA transcripts were normalized against *UBIQUITIN5*. Error bars represent standard error. A, B, C, and D refer to R1, R2, R3, and R4 respectively.

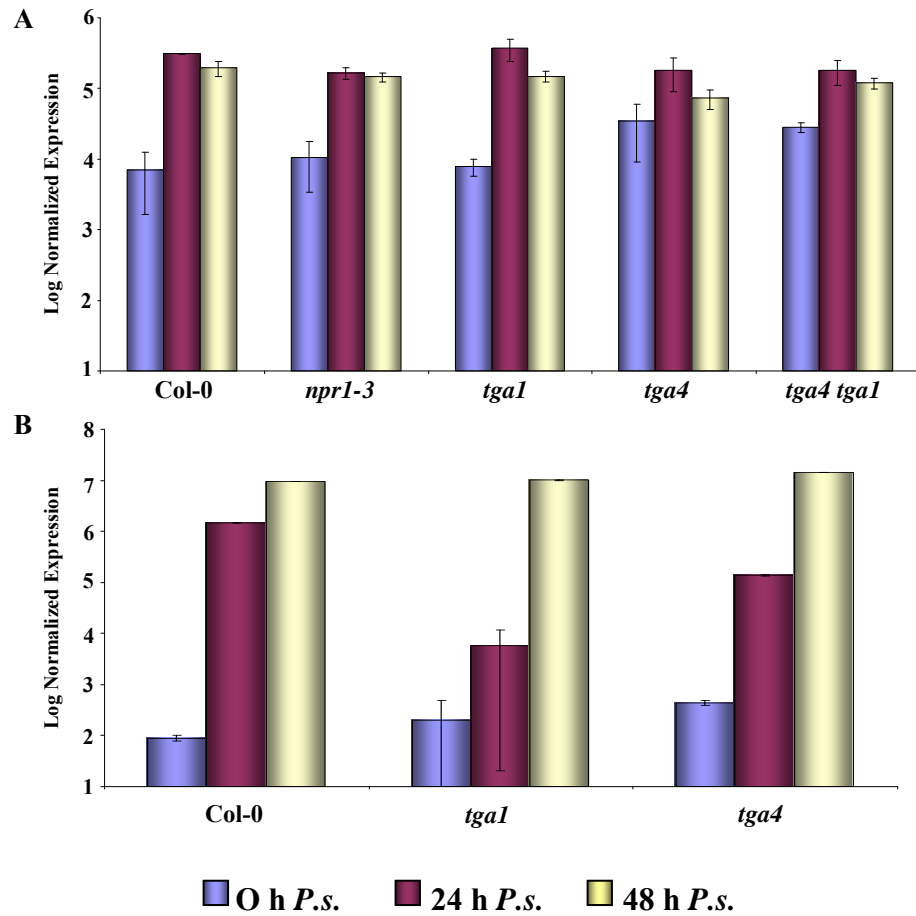


Figure 4.6 Analysis of *CXXS13* Expression Following Treatment with *Pseudomonas syringae* by qRT-PCR.

Changes in mRNA levels were assayed over a time course after *P. syringae* treatment. Each bar represents 3 biological replicates, with 4 qRT-PCR technical replicates (A) or 2 technical replicates (B) represented as the average of biological replicates. The numbers of RNA transcripts were normalized against *UBIQUITIN5*. Error bars represent standard error. A and B refer to R1 and R3 respectively.

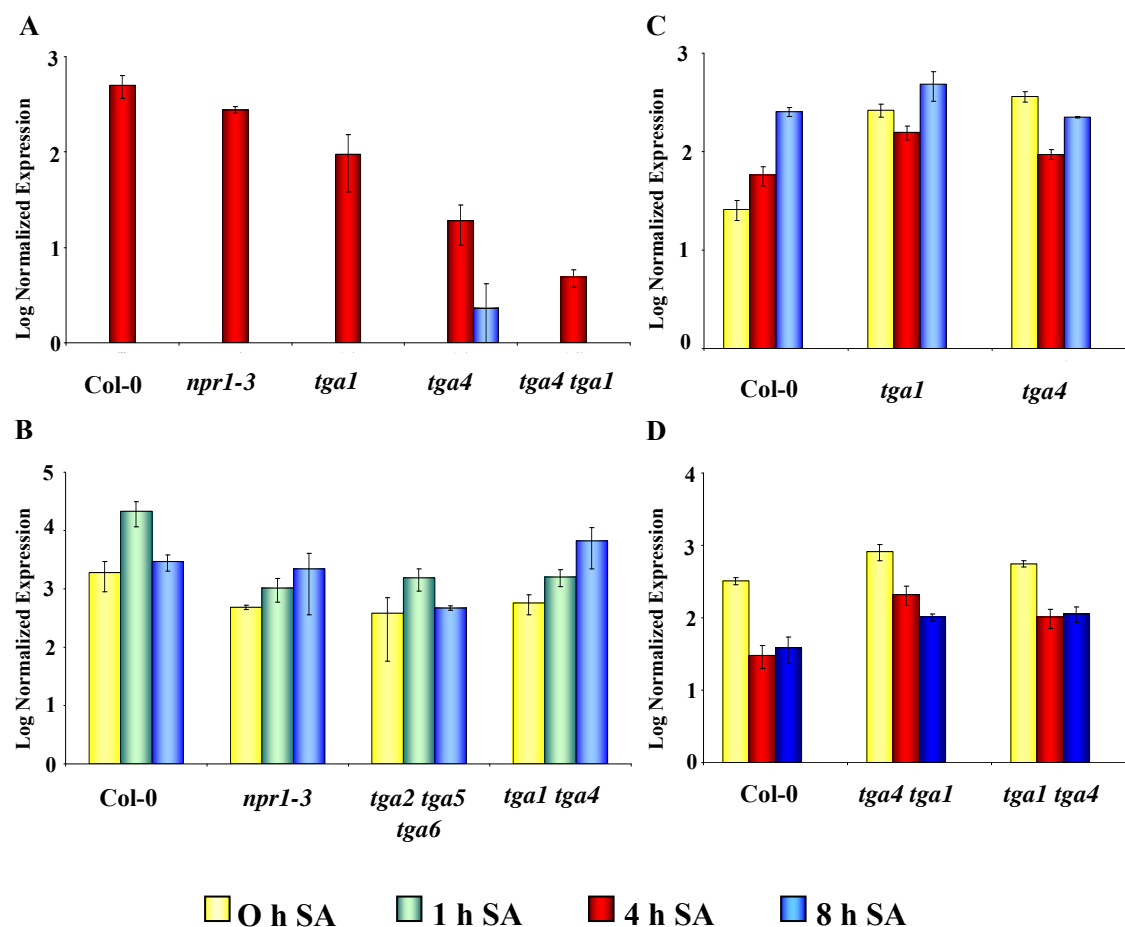


Figure 4.7 Analysis of *CXXC8* Expression Following Treatment with Salicylic Acid by qRT-PCR.

Changes in mRNA levels were assayed over a time course after SA treatment. Each bar represents 3 biological replicates, with 4 qRT-PCR technical replicates (A) or 2 technical replicates (B, C, D) represented as the average of biological replicates. The numbers of RNA transcripts were normalized against *UBIQUITIN5*. Error bars represent standard error. A, B, C, and D refer to R1, R2, R3, and R4 respectively.

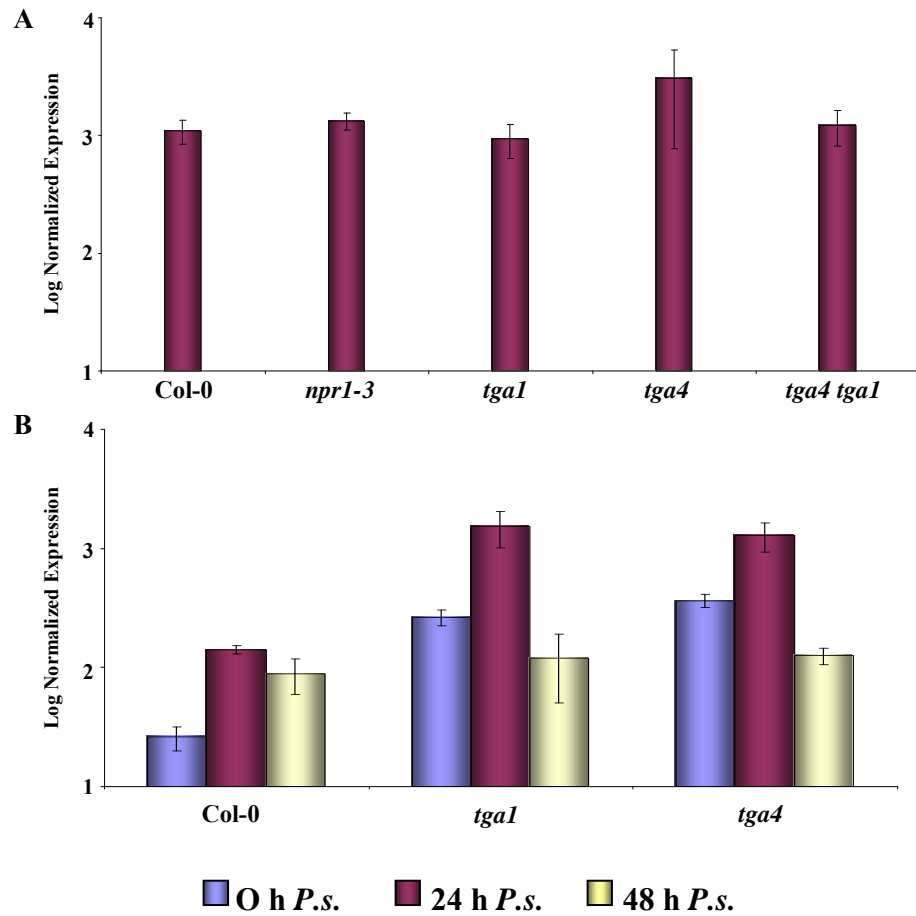


Figure 4.8 Analysis of CXXC8 Expression Following Treatment with *Pseudomonas syringae* by qRT-PCR.

Changes in mRNA levels were assayed over a time course after *P. syringae* treatment. Each bar represents 3 biological replicates, with 4 qRT-PCR technical replicates (A) or 2 technical replicates (B) represented as the average of biological replicates. The numbers of RNA transcripts were normalized against *UBIQUITIN5*. Error bars represent standard error. A and B refer to R1 and R3 respectively.

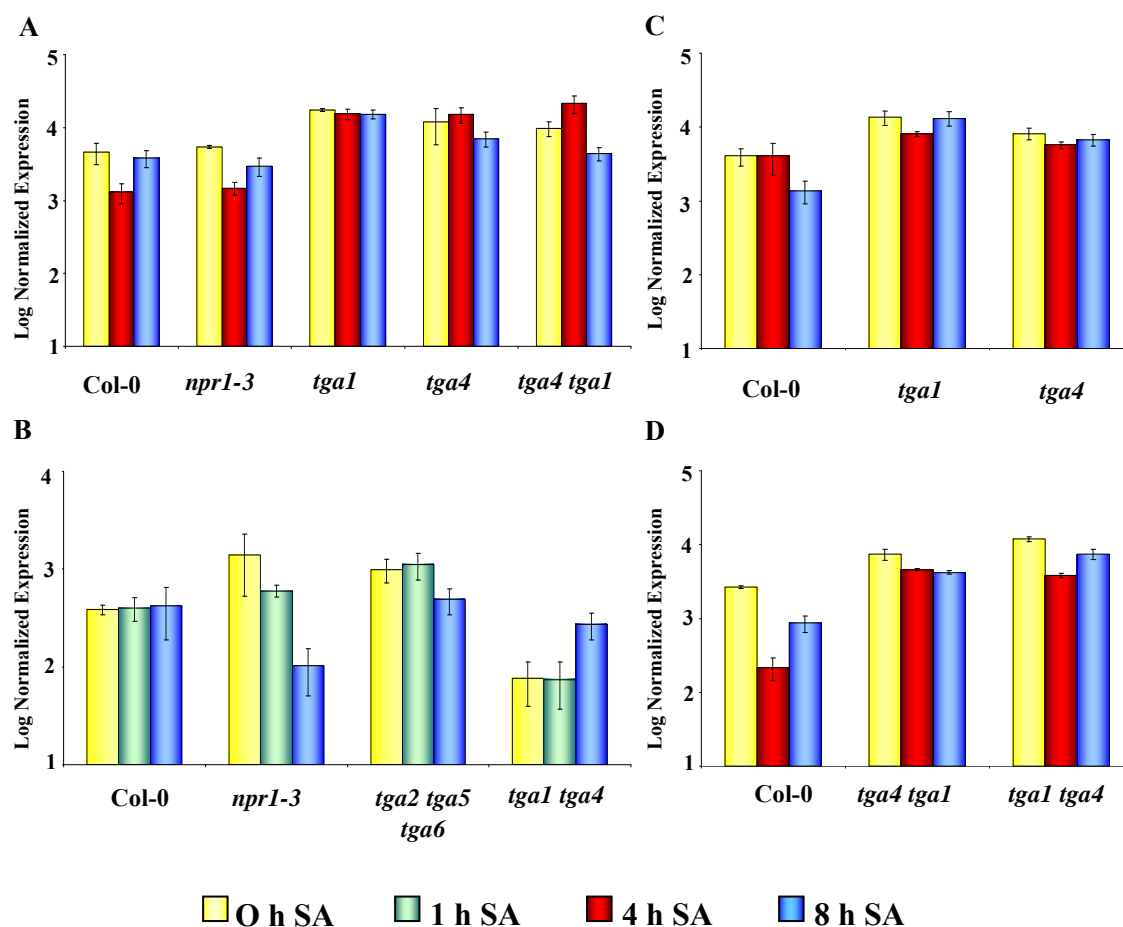


Figure 4.9 Analysis of *CXXS8* Expression Following Treatment with Salicylic Acid by qRT-PCR.

Changes in mRNA levels were assayed over a time course after SA treatment. Each bar represents 3 biological replicates, with 4 qRT-PCR technical replicates (A) or 2 technical replicates (B, C, D) represented as the average of biological replicates. The numbers of RNA transcripts were normalized against *UBIQUITIN5*. Error bars represent standard error. A, B, C, and D refer to R1, R2, R3, and R4 respectively.

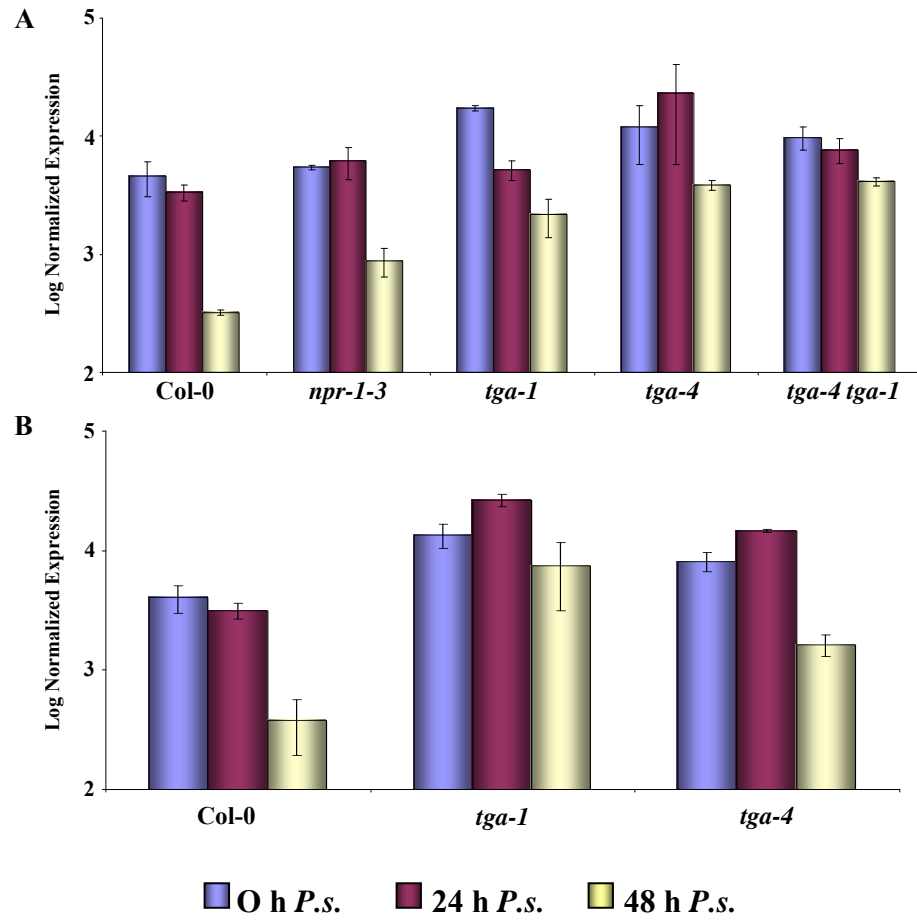


Figure 4.10 Analysis of CXXS8 Expression Following Treatment with *Pseudomonas syringae* by qRT-PCR.

Changes in mRNA levels were assayed over a time course after *P. syringae* treatment. Each bar represents 3 biological replicates, with 4 qRT-PCR technical replicates (A) or 2 technical replicates (B) represented as the average of biological replicates. The numbers of RNA transcripts were normalized against *UBIQUITIN5*. Error bars represent standard error. A and B refer to R1 and R3 respectively.



Table 4.2 Fold Changes of Transcript Levels in Mutant Leaves.

A cut off value of 2 fold increased or decreased transcript levels were set arbitrarily to indicate changes in transcript levels. Green highlight indicates increase in transcript levels, over wild type leaves, after treatment with SA or *P. syringae*. Red highlight indicates decrease in transcript levels, below wild type leaves, after treatment with SA or *P. syringae*. No highlight indicates no change in transcript levels, from wild type leaves, after treatment with SA or *P. syringae*. Grey highlight indicated no data for a replicate set. R1, R2, R3, and R4 refer to replicate 1, replicate 2, ect. h = hour.

		SA				<i>P. syringae</i>		
	PR1	0 h	4 h	8 h		0 h	24 h	48 h
R1	<i>npr-1</i>	36.81	-12.91	-184.20		36.81	-170.91	-10.83
	<i>tga-1</i>	12.29	3.38	21.21		12.29	1.10	-1.85
	<i>tga-4</i>	61.61	15.10	17.85		61.61	-1.02	-3.31
	<i>tga-1 tga-4</i>	4.79	16.38	41.98		4.79	-1.41	-1.68
		0 h	1 h	8 h				
R2	<i>npr-1</i>	-56.71	-144.00	-3741.88				
	<i>tga2 tga-5 tga-6</i>	-8.64	1.48	-1.16				
	<i>tga-1 tga-4</i>	11.06	50.22	1.79				
		0 h	4 h	8 h		0 h	24 h	48 h
R3	<i>tga-1</i>	2.24	-2.80	1.01		2.24	-250.60	1.07
	<i>tga-4</i>	4.97	4.16	-1.01		4.97	-10.51	1.53
		0 h	4 h	8 h				
R4	<i>tga-4 tga-1</i>	1.34	4.52	2.22				
	<i>tga-1 tga-4</i>	1.34	1.57	1.99				
	CXXC9	0 h	4 h	8 h		0 h	24 h	48 h
R1	<i>npr-1</i>	2.51	-4.41	-2.09		2.51	1.21	-2.01
	<i>tga-1</i>	-1.69	5.12	8.13		-0.59	1.49	-1.19
	<i>tga-4</i>	-1.44	10.52	5.42		-0.69	-45.00	1.08
	<i>tga-1 tga-4</i>	-2.20	17.37	3.64		-0.45	1.29	-1.36
		0 h	1 h	8 h				
R2	<i>npr-1</i>	-1.31	-7.06	-11.31				
	<i>tga2 tga-5 tga-6</i>	-4.12	-1.50	-9.50				
	<i>tga-1 tga-4</i>	1.21	1.55	-1.36				
		0 h	4 h	8 h		0 h	24 h	48 h
R3	<i>tga-1</i>	-0.92	-1.68	-1.95		-0.92	-3.35	1.34
	<i>tga-4</i>	3.59	4.58	-1.52		3.59	-2.96	1.29
		0 h	4 h	8 h				
R4	<i>tga-4 tga-1</i>	-19.69	1.64	1.37				
	<i>tga-1 tga-4</i>	-2.83	-1.75	1.88				
	CXXS13	0 h	4 h	8 h		0 h	24 h	48 h
R1	<i>npr-1</i>	1.51	-3.09	-1.01		1.51	-1.86	-1.33

	<i>tga-1</i>	1.10	2.01	4.10		1.10	1.19	-1.30
	<i>tga-4</i>	4.86	2.34	1.78		4.86	-1.72	-2.66
	<i>tga-1 tga-4</i>	4.04	3.03	1.97		4.04	-1.72	-1.62
		0 h	1 h	8 h				
R2	<i>npr-1</i>	1.37	-7.51	-9.55				
	<i>tga2 tga-5 tga-6</i>	-2.34	-4.43	-2.65				
	<i>tga-1 tga-4</i>	1.85	1.88	-1.24				
		0 h	4 h	8 h		0 h	24 h	48 h
R3	<i>tga-1</i>	7.89	1.15	1.04		7.89	-2.48	4.67
	<i>tga-4</i>	2.48	4.88	-0.93		2.48	-1.23	4.79
		0 h	4 h	8 h				
R4	<i>tga-4 tga-1</i>	4.72	9.96	9.57				
	<i>tga-1 tga-4</i>	11.46	5.46	8.30				
	CXXC8	0 h	4 h	8 h		0 h	24 h	48 h
R1	<i>npr-1</i>	0.00	-1.82	-1.19		0.00	1.20	0.00
	<i>tga-1</i>	0.00	-5.23	1.95		0.00	-0.85	0.00
	<i>tga-4</i>	0.00	-26.08	12.13		0.00	2.79	0.00
	<i>tga-1 tga-4</i>	0.00	-103.11	1.51		0.00	1.11	0.00
		0 h	1 h	8 h				
R2	<i>npr-1</i>	-3.98	-20.48	-1.33				
	<i>tga2 tga-5 tga-6</i>	-4.98	-13.77	-6.16				
	<i>tga-1 tga-4</i>	-3.36	-13.22	2.31				
		0 h	4 h	8 h		0 h	24 h	48 h
R3	<i>tga-1</i>	-10.17	-2.71	-1.92		10.17	10.90	1.36
	<i>tga-4</i>	-14.04	-1.63	1.13		14.04	9.14	1.41
		0 h	4 h	8 h				
R4	<i>tga-4 tga-1</i>	2.52	6.87	2.65				
	<i>tga-1 tga-4</i>	1.73	3.34	2.97				
	CXXS8	0 h	4 h	8 h		0 h	24 h	48 h
R1	<i>npr-1</i>	1.19	1.14	-1.30		1.19	1.83	2.76
	<i>tga-1</i>	3.80	11.88	4.01		3.80	1.55	6.78
	<i>tga-4</i>	2.63	11.56	1.85		2.63	6.89	12.09
	<i>tga-1 tga-4</i>	2.13	16.39	1.16		2.13	2.29	12.92
		0 h	1 h	8 h				
R2	<i>npr-1</i>	3.66	1.49	-4.13				
	<i>tga2 tga-5 tga-6</i>	2.56	2.76	1.16				
	<i>tga-1 tga-4</i>	-5.06	-5.41	-1.56				
		0 h	4 h	8 h		0 h	24 h	48 h
R3	<i>tga-1</i>	3.34	1.97	9.55		3.34	8.39	19.54
	<i>tga-4</i>	2.01	1.39	4.89		2.01	4.62	4.30
		0 h	4 h	8 h				
R4	<i>tga-4 tga-1</i>	2.79	21.16	4.87				
	<i>tga-1 tga-4</i>	4.49	17.64	8.68				

### *Expression of PR-1*

*PR-1* expression in Col-0 is upregulated in all repetitions (experimental repetitions will be denoted as R1, R2 etc.) 4 h after SA treatment or 24 h after inoculation using a virulent strain of the bacterial pathogen *P. syringae* (Figures 4.1 & 4.2, Table 4.1). Transcript levels subsequently remain elevated after the 8 h SA or 48 h *P. syringae* time points (Figures 4.1 & 4.2, Table 4.1). The greatest increases in *PR-1* transcripts (~1,000 to 10,000 fold) were observed following *P. syringae* infection (Figure 4.2, Table 4.1). Increases following SA treatment were generally more modest (~25 to 2,000 fold), although an approximately 12,000-fold increase was observed in R3 at the 8 h time point (Figure 4.1, Table 4.1). One hour following SA treatment was insufficient to increase levels of *PR-1* transcripts (Figure 4.1 B). The greatest increases in *PR-1* transcript following SA treatment were observed in R3 followed by R4. The smallest increase was found in R1. In contrast, R1 showed the greatest increase of *PR-1* following *P. syringae* infection. Thus, in all four repetitions *PR-1* was consistently induced by SA and *P. syringae*, indicating that there is an effective mounting of the defense response in the biological material.

### *Expression of CXXC9*

*CXXC9* transcript levels in Col-0 increased 9 to 95-fold after treatment with SA and 34 to 466-fold after infection with *P. syringae* (Figures 4.3 & 4.4, Table 4.1). As witnessed in R2, 1 h following SA treatment did not appear sufficient to increase *CXXC9* transcript levels, yet the highest levels of mRNA were observed by 4 h in R1, R3 and R4. Levels at 8 h remained similar (R1) or decreased slightly compared to 4 h (R3, R4). In leaves infected with *P. syringae*, *CXXC9* transcripts continued to increase

between 24 and 48 h in R1 but remained similar between these time points in R3. Increases of *CXXC9* levels following *P. syringae* challenge were greater in R3 than R1.

#### *Expression of CXXS13*

Transcripts of *CXXS13* in Col-0 increased following SA treatment and *P. syringae* infection (Figures 4.5 & 4.6, Table 4.1). Increases following SA treatment were lowest in R1 and R4 (4-9 fold) and greatest in R3 (140-791). In R3, 8 h at SA treatment was required for the highest increases, while in R1 and R4, levels observed at 4 and 8 h were similar. In leaves infected with *P. syringae*, *CXXS13* transcript levels were 27-460 fold higher than the uninduced samples at 24 h and 48 h in both repetitions. Increases of *CXXS13* levels following *P. syringae* challenge were about 10 times greater in R3 than R1. Thus, for *PR-1*, *CXXC9* and *CXXS13*, the greatest increases in transcript levels were observed in R3 and the smallest increases were observed in R1.

#### *Expression of CXXC8*

The expression of *CXXC8* in R1 leaves of Col-0 treated with SA is transient; at 4 h it is 500 times higher than in the untreated leaves, while at 8 h it is below the threshold level of detection (i.e. no Ct obtained) (Figure 4.7, Table 4.1). The expression of *CXXC8* in R1 leaves treated with *P. syringae* is also transiently upregulated 1100 fold at 24 h, and returns to zero at 48 h (Figure 4.8, Table 4.1). In R2 leaves treated with SA the expression is again somewhat transient being 11 times higher at 1 h than in the untreated leaves, and a subsequent 7-fold decrease by 8 h. Repetition 3 and R4 leaves treated with SA do not follow similar patterns. In R3 treated leaves the expression at 4 h is just over 2 times higher than in the untreated leaves and is further upregulated 4.5 times at 8 h. In R3 leaves treated with *P. syringae* there is a 5-fold increase in transcript

level of *CXXC8* at 24 h compared to untreated leaves and is then downregulated from this level by 1.5-fold at 48 h. In R4 leaves there is an 11 fold decrease at 4 h compared to untreated, and transcript levels remain at this low level at 8 h.

#### *Expression of CXXS8*

The expression of *CXXS8* does not appear to be upregulated by SA or *P. syringae* in Col-0 leaves. In fact, transcript levels of this Grx-like gene typically decrease following the different treatments tested. In R1, R3 and R4, leaves treated with SA displayed reduced levels of *CXXS8* transcript (Figure 4.9, Table 4.1). In R1, reduction was only observed at 4 h, while in R3 reduction was only observed at 8 h. The expression of *CXXS8* in leaves infected with *P. syringae* is almost identical in both replicates; no differences in transcript levels were detectable at 24 h, and levels were reduced compared to untreated leaves at 48 h (Figure 4.10, Table 4.1).

Thus, for Col-0, in all repetitions, transcripts of the CC-type Grxs *CXXC9*, *CXXS13*, and *CXXC8* increased in response to both SA and *P. syringae*, while levels of *CXXS8* were reduced by these treatments. However, there was substantial difference in the absolute levels of transcript and in fold changes measured in the separate repetitions.

#### **4.1.1 Expression of Glutaredoxins-Like Genes in Response to Genotype Effects**

As noted above, microarray analysis indicated that transcripts of genes encoding several Grx-like proteins were upregulated in leaves of *tga1-1 tga4-1* double mutant plants (Fobert et al. unpublished). To confirm and extend upon these results, levels of *PR-1* and Grx-like transcripts were quantified in the *tga1-1 tga4-1* (and its reciprocal

*tga4-1 tga1-1*) double mutants, the *tga1-1* and *tga4-1* single mutants, *npr1-3*, and the triple mutant *tga2-1 tga5-1 tga6-1* plants.

#### *Expression of PR-1*

In the absence of SA or *P. syringae* treatment, *PR-1* levels were higher in leaves of the various clade I TGA factor mutants than in the Col-0 wild-type (Figure 4.1, & Figure 4.2, Table 4.2). This was most evident in R1, R2 and R3 where levels of *PR-1* were between 4-60 fold higher, while R4 showed no change in expression. Following treatment with SA, *PR-1* levels remained elevated above those measured in the wild-type at 8 h by 2-40 fold in R1, R3 and R4. In R2 expression peaked in leaves at 1 h by 50 fold over wild type levels (Figure 4.1, Table 4.2). There was no consistent trend with respect to differences of *PR-1* transcripts between wild type and TGA clade I mutants in leaves treated with *P. syringae*. In R1, levels were unchanged or mildly reduced in the TGA mutants compared to wild type, while in R3 levels were downregulated in both the *tga1-1* and *tga4-1* mutants at 24 h (Figure 4.2, Table 4.2), while they were unchanged from wild-type at 48 h.

As indicated previously, *PR-1* is expressed in response to SA or *P. syringae* (Durrant and Dong, 2004). It also has a distinct expression pattern in various mutants, particularly in *npr1* mutants, where transcript levels are severely reduced in response to SA and pathogen (Cao et al., 1994; Delaney et al., 1995; Wang et al., 2005; Mosher et al., 2006). Consistent with the literature, steady-state levels of *PR-1* were found to be downregulated in leaves of the *npr1-3* mutant at treatment with SA or *P. syringae* (Figure 4.1, & Figure 4.2, Table 4.1). Reductions of up to ~200-3000 fold were

observed in SA and *P. syringae* treated leaves at 8 h and 48 h, respectively (Figure 4.1, & Figure 4.2, Table 4.2).

The *tga2-1 tga5-1 tga6-1* mutant reportedly displays a similar expression pattern of *PR-1* as the *npr1-1* mutant (Zhang et al., 2003). Prior to treatment with SA, *PR-1* levels were 9-fold lower in mutant leaves than the wild-type (Figure 4.1, Table 4.2). However, after treatment of leaves with SA levels of *PR-1* rapidly increased to levels similar to those observed in the wild-type.

#### *Expression of CXXC9*

In the TGA 1 clade mutants prior to treatment with SA or *P. syringae* (Figure 4.3, & Figure 4.4, Table 4.2) there is no consistent difference in transcript levels compared to the wild-type. After treatment of leaves with SA, transcript levels of *CXXC9* in TGA1 clade mutants were found to vary, from 9-fold lower (*tga4-1* in R2) all the way to 17-fold higher (*tga4-1 tga1-1* in R1) than wild-type across all four repetitions (Figure 4.3, Table 4.2). Only in R1 did levels of *CXXC9* display a consistent increase in TGA clade I mutants over corresponding wild-type treatments. Leaves collected at 24 h following treatment with *P. syringae* had decreased transcript levels (2-45 fold lower than corresponding wild type) in both R1 and R3 (Figure 4.4, Table 4.2). There was no difference from wild-type in transcript levels in leaves collected at 48 h.

In the *npr1-3* mutants the level of *CXXC9* transcript prior to treatment with SA or *P. syringae* varied from wild-type; R1 has a 2-fold increase in transcript levels over wild-type, while R2 is no different from wild-type (Figure 4.3, & Figure 4.4, Table 4.2).

After treatment with SA or *P. syringae* in *npr1-3* leaves there is either no difference in transcript levels of *CXXC9* or the levels are slightly decreased compared to wild type.

In the leaves of the *tga2-1 tga5-1 tga6-1* mutant prior to treatment with SA or (Figure 4.3, Table 4.2) there is a 4-fold decrease in transcript levels of *CXXC9* compared to wild-type. After treatment with SA, levels of transcript remains reduced by up to 9-fold at 8 h compared to wild-type.

#### *Expression of CXXS13*

The levels of *CXXS13* RNA transcript in mutants of the clade 1 TGA factors prior to treatment with SA or *P. syringae* (Figure 4.5 & Figure 4.6, Table 4.2) are higher than wild-type in all four replicates. This is most apparent in R1 for the *tga4-1* and *tga4-1 tga1-1* mutants and R4. After treatment with SA the level of transcripts reveals no consistent differences relative to the corresponding wild-type time points. Higher levels are observed in R1 and R4, but not in R2 or R3, (Figure 4.6, Table 4.2). No consistent trend is observed after treatment with *P. syringae*, although a decrease in levels at the 24 h time point in R3 was noted (Figure 4.6, Table 4.2)

In the *npr1-3* mutant leaves treated with SA levels of *CXXS13* transcript are lower than those in wild-type at the corresponding time only in R2 (Figure 4.5, Table 4.2). This mutant shows no change in *CXXS13* levels following challenge with *P. syringae* (Figure 4.6, Table 4.2).

In the *tga2-1 tga5-1 tga6-1* mutant leaves before treatment with SA (Figure 4.5, Table 4.2) a 2-fold decrease in expression compared to wild-type was observed. After treatment with SA levels of *CXXS13* transcript in this mutant leaves remain below wild-type with SA levels by up to 4-fold at the 1 h and 8 h time points.



### *Expression of CXXC8*

Comparisons of *CXXC8* transcript levels between wild-type and the TGA1 clade mutant leaves before treatment with SA or *P. syringae* revealed no consistent trends (Figure 4.7 & Figure 4.8, Table 4.2). Levels of transcripts in TGA1 clade mutants run the gamut of possibilities; undetectable, higher, and lower levels than wild-type levels (Figure 4.7, & Figure 4.8, Table 4.2). Similarly, after treatment with SA or *P. syringae* comparisons reveal no consistent trends in relative changes of transcript levels, although generally lower levels were observed in TGA1 clade mutants in R1 (Figure 4.7 & Figure 4.8, Table 4.2).

*CXXC8* transcripts were not detectable in wild-type and *npr1-3* mutant leaves prior to treatment with SA or *P. syringae* in R1, making comparisons difficult (Figure 4.7, Table 4.1). In R2, transcripts levels of this Grx-like gene are lower in the *npr1-3* mutant without treatment. In R1 4 h following SA treatment and in R3 at both 1 h and 8 h after SA, *CXXC8* levels are lower than corresponding wild-type values (Figure 4.7, Table 4.2). Twenty-four hours after challenge with *P. syringae* levels of *CXXC8* are similar in the *npr1-3* mutant and wild-type. Transcript is not detectable in either genotype at the 48 h time point (Figure 4.8, Table 4.1).

In the *tga2-1 tga5-1 tga6-1* mutant leaves prior to treatment with SA, transcript levels of *CXXC8* are 5-fold below wild-type levels (Figure 4.7 Table 4.1). After treatment with SA levels of transcript remains lower than wild-type at 8 h (Figure 4.7 Table 4.2).

### *Expression of CXXS8*

Levels of *CXXS8* transcript in leaves of TGA1 clade mutants prior to treatment with SA or *P. syringae* are 2-11 fold higher than wild-type in all four repetitions, with the exception of the *tga1-1 tga4-1* double mutant in R2, where levels are lower (Figure 4.9 & Figure 4.10, Table 4.2). After treatment of leaves with SA or *P. syringae* transcript levels remain higher than wild-type levels by 2-50 fold except in R2, where they are lower than wild-type after SA treatment (Figure 4.9 & Figure 4.10, Table 4.1).

In the *npr1-3* mutants leaves before and after treatment with SA or *P. syringae* have stark contrasts in transcript accumulation between the repetitions (Figure 4.9 & Figure 4.10, Table 4.2). In R1 leaves treated with either SA or *P. syringae* the accumulation of transcript *CXXS8* in the *npr1-3* is no different from wild-type. However, in R2 accumulation of transcript is 5-fold lower than wild-type prior to treatment of leaves with SA, and is over 4-fold below wild-type levels at 8 h.

In the leaves of the *tga2-1 tga5-1 tga6-1* mutant transcript levels are 2.5-fold than in wild-type prior to treatment with SA (Figure 4.9, Table 4.1). Eight hours after treatment of leaves with SA there is no difference from wild type expression.

#### **4.1.2 Expression of Glutaredoxin-Like Genes in Response to Jasmonic Acid**

A recent publication reported that *CXXC9* was induced in response to jasmonic acid (JA) (Ndamukong et al., 2007). Furthermore, plants that overexpress *CXXC9* were found to have reduced expression of the JA pathway marker gene *PDF1.2*. Accordingly, experiments were designed to determine whether other Grx-like genes

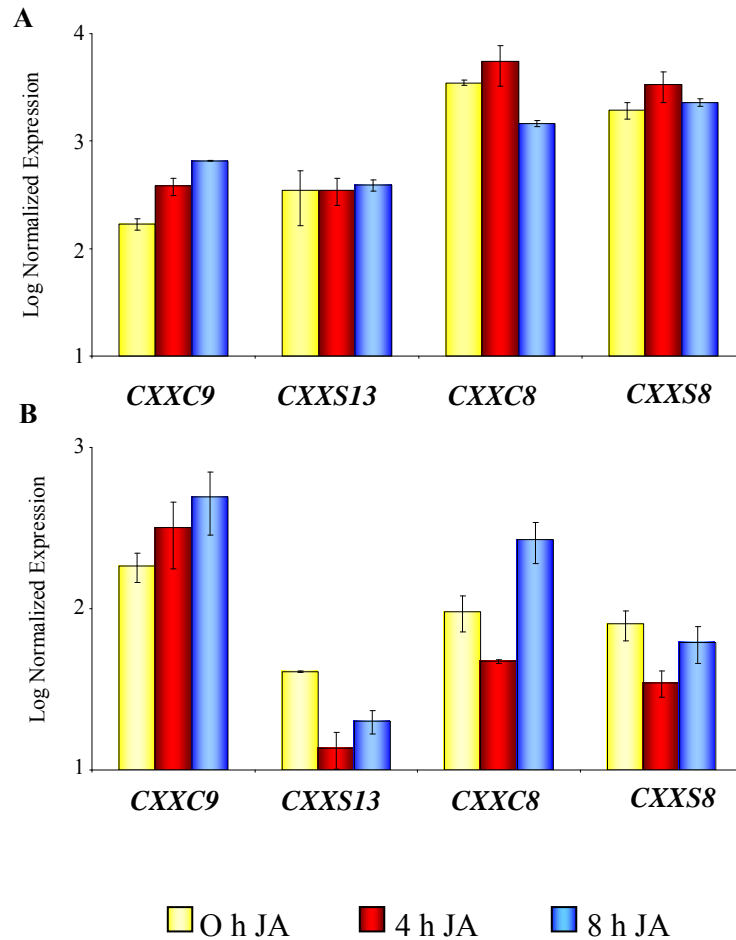


Figure 4.11 Analysis of Glutaredoxin-Like Transcript Abundance in Wild Type *Arabidopsis* Following Treatment with Methyl Jasmonate by qRT-PCR.

Error bars represent standard error. A, and B = R5, and R6 respectively. Changes in mRNA levels were assayed over a time course after JA treatment. Each bar represents the average of 3 biological replicates, with 2 technical replicates. The numbers of RNA transcripts were normalized against *UBIQUITIN5*. Error bars represent standard error. A and B represent repetitions R5 and R6 respectively.

Table 4.3 Fold Changes of Glutaredoxin-Like Transcript Levels in Response to Jasmonic Acid

A cut off value of 2 fold increased or decreased transcript levels were set arbitrarily to indicate changes in transcript levels. Green highlight indicates increase in transcript levels, over wild type leaves, after treatment with JA. No highlight indicates no change in transcript levels, from wild type leaves, after treatment with SA or *P. syringae*. R5 and R6 refer to replicate 5 and replicate 6.

	R5		R6	
JA	Fold change 4 h	Fold change 8 h	Fold change 4 h	Fold change 8 h
CXXC9	2.25	3.87	1.72	2.69
CXXS13	1.00	1.12	-3.00	-2.04
CXXC8	1.58	-2.39	-2.04	2.78
CXXS8	1.72	1.18	-2.31	-1.30
PDF1.2	2.77	9.61	1.57	4.14

follow a similar pattern of transcript accumulation and to confirm the results of Ndamukong et al. (2007).

In two separate experiments (R5 and R6, to distinguish from previous SA and pathogen repetitions) leaves treated with methyl JA (MeJA) displayed 3-4-fold higher levels of *CXXC9* transcript than untreated leaves (Figure 4.11, Table 4.3). None of the other Grx-like genes show a consistent pattern of transcript accumulation between the two repetitions. Furthermore, none other than *CXXC9* show increased levels of transcript in response to MeJA, with the exception of *CXXC8*, which is upregulated at 8 h MeJA treatment in R6 only. In some cases, levels of transcripts were downregulated in response to MeJA (e.g. *CXXS13* and *CXXS8* in R6). However, this was not consistently observed between repetitions. Accordingly, *CXXC9* appears to be the only Grx-like transcript, of the ones tested by qRT-PCR, that is inducible by MeJA in addition to SA and *P. syringae*. Transcripts of the other Grx-like genes, *CXXS13*, *CXXC8*, and *CXXS8* do not follow a consistent pattern of expression in response to MeJA in the two repetitions.

#### **4.2 Expression of *PDF-1.2* in *Arabidopsis* Plants with Altered Expression of Glutaredoxin-Like Genes**

In order to confirm whether over expression of *CXXC9* in plants results in repression of *PDF1.2*, as reported in Ndamokong et al. (2007), two lines expressing a CaMV 35S:*CXXC9* transgene and T-DNA insertion lines of *CXXC9* and *CXXS13* were analyzed for reporter gene expression, response to oxidative stress and resistance to a bacterial pathogen.

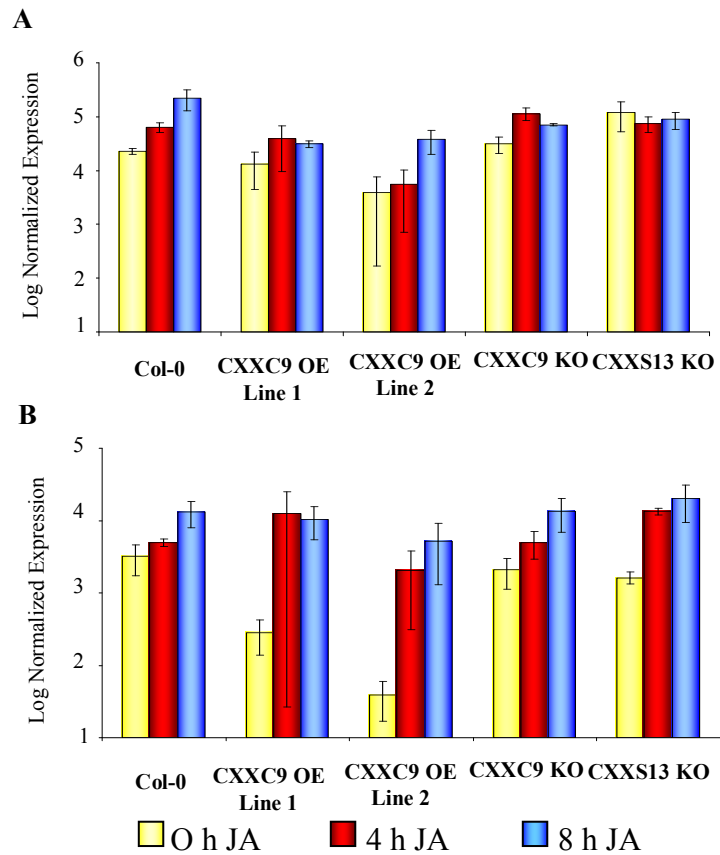


Figure 4.12 Analysis of *PDF1.2* Expression Following Treatment with Methyl Jasmonate by qRT-PCR.

Changes in mRNA levels were assayed over a time course after JA treatment. Each bar represents the average of 3 biological replicates, each analyzed twice (i.e. 2 technical replicates). The numbers of RNA transcripts were then normalized against *UBIQUITIN5*. Error bars represent standard error. A and B represent repetitions R5 and R6 respectively. KO, T-DNA insertion (knock out) mutant; OE, independent over expression line.

### *Expression of PDF1.2*

In wild-type plants (Col-0), expression of *PDF1.2* has been reported to increase after exogenous application of MeJA (Pennickx et al., 1998; Thomma et al., 1998). In both R5 and R6, levels of *PDF1.2* transcript in wild-type leaves increased 4-9 fold 8 h after spraying with MeJA (Figure 4.12, Table 4.3). Plants in R5 have increased accumulation of *PDF1.2* 4 h after spraying leaves with MeJA, whereas in R6 there is no difference detected at this time point.

In line 1 of CaMV:35SCXXC9 levels of *PDF1.2* transcript do not accumulate in a consistent pattern, either prior to or after treatment with MeJA. In R5, there is no difference from wild-type in transcript levels in untreated leaves and 4 h after treatment with MeJA, while at 8 h transcript levels are 7- fold lower than wild type. In R6 levels of *PDF1.2* in this line are lower than wild-type in the absence of MeJA, but there is difference in expression levels 8 h after MeJA treatment (Figure 4.12, Table 4.3). However in line 2 of CaMV:35SCXXC9, accumulation of *PDF1.2* transcript is 5-15 fold lower than wild type, both prior to and after treatment of plants with MeJA, and in both R5 and R6 (Figure 4.12, table 4.3). Taken together, these results indicate that over expression of CXXC9 suppresses MeJA-induced expression of *PDF1.2*

In both the CXXC9 and CXXS13 T-DNA insertion lines there is no apparent difference in the accumulation of *PDF1.2* transcript either before or after spraying plants with MeJA in either repetition. Interestingly, as noted in the previous section on expression of Grx-like genes in response to MeJA (4.1.3), the overall accumulation of *PDF1.2* transcripts in all five genotypes is on average 36 times higher in R5 than in R6.

The largest differences in transcript accumulation were generally found in untreated leaves and ranged from 7-342 fold higher in R5 than in R6.

#### **4.2.1 Tolerance to Oxidative Stress in *Arabidopsis* Plants with Altered Expression of Glutaredoxin-Like Genes**

Studies conducted on yeast Grxs have revealed that overexpression of specific Grx genes results in cells that are more tolerant to exogenous exposure to oxidative stress such as H<sub>2</sub>O<sub>2</sub> (Luikenhuis et al., 1998; Collingson et al., 2002). In order to determine whether the *Arabidopsis* Grx-like proteins may be able to provide similar protection, two independent lines over expressing *CXXC9* (line #1OE and line #2OE), one T-DNA insertion mutant of *CXX9*, and one *CXXS13* T-DNA insertion mutant (*CXXC9* KO and *CXXS13* KO respectively) were grown on plates in the presence or absence of the oxidative stress-inducing chemical methyl viologen (paraquat). Root lengths of plantlets were measured on both treatment groups as an indication of how plants were able to cope with the oxidative stress.

The plant roots of all five genotypes grown on the control plates were up to 5 times longer than those grown paraquat (Figure 4.13). Additionally, growth on the control plates is consistent between all five genotypes as there was not significant difference revealed after performing a 1-way ANOVA ( $\alpha = 0.05$ ). Similar trends were observed in one other repetition; however, statistics were unable to be preformed because there were not enough replicates in the experimental setup.

Growth of plant roots on the paraquat plates reveals an interesting, and unexpected pattern in relation to the five genotypes. Only two genotypes were found to



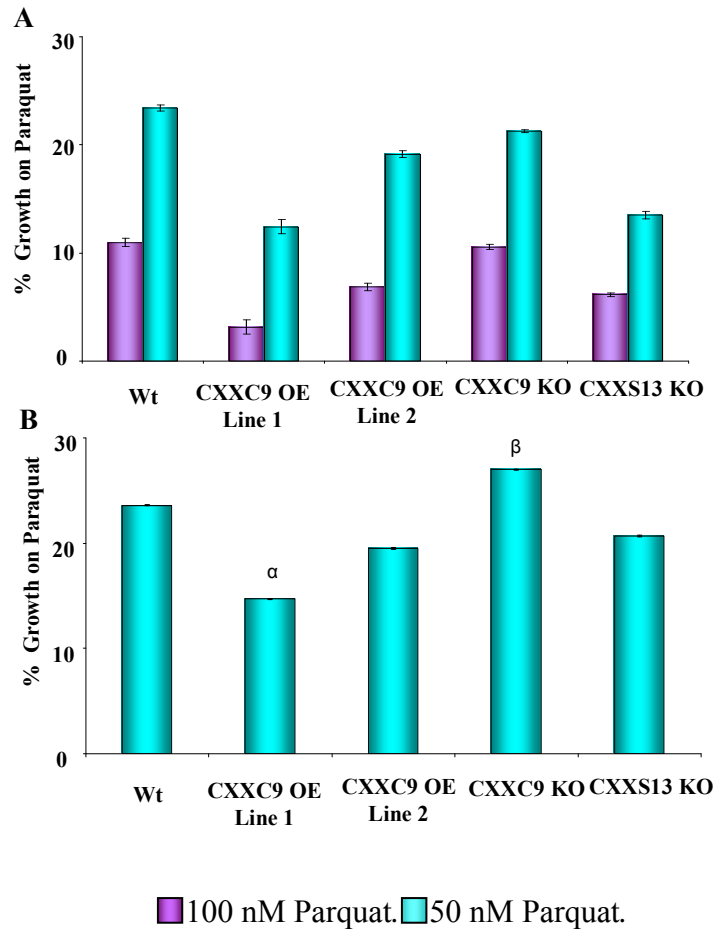


Figure 4.13 Effect of Oxidative Stress in *Arabidopsis* Plants with Altered Glutaredoxin-Like Gene Expression.

Plants were grown on agrose Murashige and Skoog plates with or without paraquat. Plant roots were measured, 13 technical replicates per plate with 1 (A) or 4 (B) biological replicates. Percent growth on paraquat was determined by dividing the growth observed on paraquat plates by the growth observed on control plates and multiplying by 100. A 1-way ANOVA was performed on (B)  $\alpha$  and  $\beta$  indicate statistical differences. KO, T-DNA insertion (knock out) mutant; OE, independent over expression line.

have significantly different root length growth from the wild type in 1-way ANOVA ( $\alpha = 0.05$ ) and Tukey's tests; line #1 *CXXC9* OE plants and the *CXXC9* KO (Figure 4.13). Interestingly, line #1 *CXXC9* OE plants have shorter roots than wild type, while the *CXXC9* KO plants have longer roots than wild type. Although the line #2 *CXXC9* OE plants also have shorter roots than wild type the Tukey's test did not identify this difference as significant. The *CXXS13* KO plants have slightly shorter roots than wild type plants, but the difference was not significant. Similar trends were also observed in an additional repetition. The additional repetition also contained an extra paraquat treatment at a higher concentration. Increasing the paraquat concentration does not alter the response to oxidative stress, as the same trend of root growth is observed; however, these plants were much smaller than the control and at times difficult to measure accurately. Thus, overexpression of *CXXC9* decreases the plants tolerance to oxidative stress as is evident by shorter root lengths. Conversely knocking out *CXXC9* increases the tolerance to oxidative stress.

#### **4.2.2 Response to *P. syringae* in *Arabidopsis* Plants with Altered Expression of Glutaredoxin-Like Genes**

Since the Grx-like transcripts are inducible by SA and pathogen challenge, it is reasonable to make inference that the encoded proteins may play a role in plant defense pathways. To test this possibility, the ability of plants to mount SAR was tested by first infiltrating two leaves on each plant with avirulent *P. syringae*, followed by challenge with virulent *P. syringae* a few days later on several of the remaining leaves. Three days after the secondary infection, titres of virulent bacteria were quantified in leaves of wild

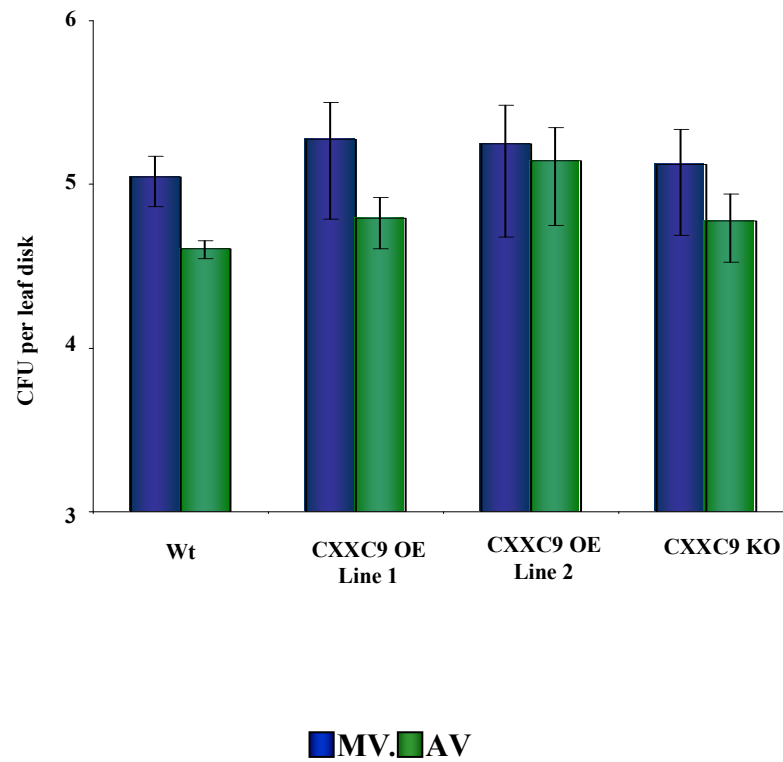


Figure 4.14 Effect of *P. syringae* in *Arabidopsis* Plants with Altered Glutaredoxin-Like Gene Expression

Each bar represents the average of 8 biological replicates, with error bars representative of standard error. MV = mock treatment (i.e. H<sub>2</sub>O), AV, avirulent pathogen.

type, line #1 *CXXC9* OE, line #2 *CXXC9* OE, and *CXXC9* KO plants. A set of control plants were pre-treated with buffer ( $\text{Mg}_2\text{Cl}$ ) instead of avirulent bacteria, followed by the secondary inoculation with virulent *P. syringae*. In this experimental design, SAR is measured as the decrease in virulent bacterial titre between the  $\text{Mg}_2\text{Cl}$  and avirulent pathogen pre-treatments. Comparing virulent bacterial titres in plants receiving the  $\text{Mg}_2\text{Cl}$  treatments between wild-type and different genotypes also provides an indication of basal resistance. After calculating the average titre of bacteria in leaves, a 2-way ANOVA was performed ( $\alpha = 0.05$ ), which indicated that there were no significant differences in bacterial growth between plants given the  $\text{Mg}_2\text{Cl}$  or avirulent pretreatments. Furthermore, there was no difference in bacterial titres between the genotypes, according to 2-way ANOVA. In addition, *t*-tests ( $\alpha = 0.05$ ) also indicated that there was no difference between the two pre-treatments in any of the four genotypes including wild type plants (Figure 4.14). Thus, altering the level of *CXXC9* (either increasing or decreasing) does not appear to have a significant effect of either SAR or basal resistance against virulent *P. syringae*.

#### **4.3 Analysis of Glutaredoxin-Like Proteins Enzymatic Activity**

As previously noted, TGA1 and members of the Grx-like protein family interact in yeast 2-hybrid assays (Fobert et al., unpublished). Previous studies revealed that TGA1 is regulated by redox conditions, and must be in its reduced form in order to interact with NPR1 (Després et al., 2003). Therefore, one possibility is that the Grx-like proteins act as the mediators of this redox regulation. To test this theory several Grx-like and one classical Grx coding sequences were individually expressed in *Escherichia*

*coli*, purified, and assayed using the  $\beta$ -hydroxyethyl disulfide (HED) assay. The HED assay is commonly used to assess the redox activity of Grxs for commercial use and whenever new and/or unique Grxs are identified (Holmgren and Åslund, 1995), as in the case with the Grx-like proteins from *Arabidopsis*.

#### 4.3.1 Protein Expression and Purification

The full-length coding regions of all genes in Table 4.4, except for CXXS8, were cloned into both the N-terminal His-tagged pRSET vector and the C-terminal His-tag pET41 vector. Several pairs of oligos were designed for CXXS8 but all failed to yield a PCR product despite several attempts using different thermocycler conditions. Cloning was initially performed in the N-terminal fusion vectors. Transformation of these vectors into *E. coli* BL21 cells did not express proteins with the expected molecular weights in adequate amounts and were extremely slow growing, even in the absence of isopropyl  $\beta$ -D-1-thiogalactopyranoside (IPTG) (data not shown). In contrast cell transformed with the C-terminal pET41 fusions grew at the doubling rate typical of *E. coli* (~ 20-30 min). All of the constructs, except for CXXS13, expressed the protein (Figure 4.15). CXXC1 was found to be soluble under native conditions, while the Grx-like proteins were insoluble (Figure 4.15 a).

In order to deal with the insolubility issue a number of methods were attempted, such as growing cells at temperatures varying from 4 °C to 36 °C, transforming in to alternative cell lines, and varying the concentration of IPTG from 0.5 mM to 3 mM. None of these methods appreciably enhanced solubility (data not shown). Lysing of cells with mild detergents (1% sarkosyl and triton X-100), followed by dialysis has been

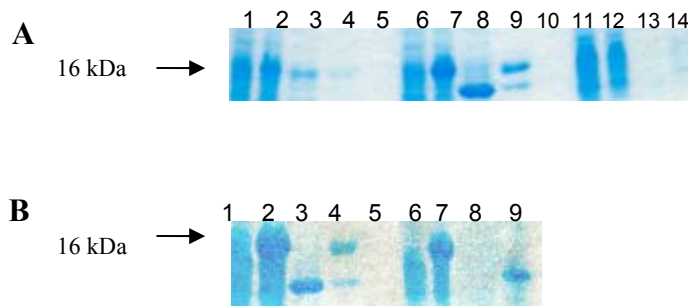


Figure 4.15. Expression of Glutaredoxin-Like Proteins

- A.** SDS Polyacrylamide gel electrophoresis (PAGE) Lanes 1-4 Expression of CXXC1; 1) whole cell extract, prior to IPTG induction, 2) 4 hour post induced whole cell extract, 3) soluble fraction from induced cells lysed by sonication, 4) insoluble fraction from induced cells lysed by sonication. Lanes 6-9 expression of CXXC8; 6) whole cell extract, prior to IPTG induction, 7) 4 hour post induced whole cell extract, 8) soluble fraction from induced cells lysed by sonication, 9) insoluble fraction from induced cells lysed by sonication. Expression of CXXS13 Lanes 11-14; 11) whole cell extract, prior to IPTG induction, 12) 4 hour post induced whole cell extract, 13) soluble fraction from induced cells lysed by sonication, 14) insoluble fraction from induced cells lysed by sonication. Lanes 5, and 10 are empty.
- B.** SDS-PAGE Lanes 1-4 expression of CXXC9; 1) whole cell extract, prior to IPTG induction, 2) 4 hour post induced whole cell extract, 3) soluble fraction from induced cells lysed by sonication, 4) insoluble fraction from induced cells lysed by sonication. Lanes 6-9 Expression of CXXC7; 6) whole cell extract, prior to IPTG induction, 7) 4 hour post induced whole cell extract, 8) Soluble fraction from induced cells lysed by sonication, 9) insoluble fraction from induced cells lysed by sonication. Lane 5 is empty.

shown to leave proteins in their native and active form (Fangioni and Neel, 1993). Accordingly, this method was tested with the Grx-like proteins. The proteins that were previously insoluble became approximately 50% soluble using the sarkosyl method based on SDS polyacrylamide gel electrophoresis (PAGE) results (Figure 4.16). Proteins were then subjected to purification using a nickel NTA purification system and a single band of protein was detected by SDS-PAGE (Figure 4.17).

### 4.3.2 $\beta$ -Hydroxyethyl Disulfide Assay

In the HED assay a spontaneous reaction occurs to form a low molecular weight disulfide between GSH and the HED substrate, whose reduction by Grx can be indirectly monitored by the decrease in absorption at 340 nm as NADPH is oxidized to  $\text{NADP}^+$  through glutathione reductase (Holmgren and Åslund, 1995). A commercially acquired *E. coli* Grx was first used to standardize the assay. The classical Grx CXXC1 was tested several times after independent purification trials by either the sarkosyl method or by standard native purification procedures, in order to assess whether these methods affected the activity of the protein. In all purification batches the activity remained constant at an average of  $153 \pm 14$ , indicating that the sarkosyl and triton-X100 did not interfere with the activity of this Grx (Table 4.4).

Two of the three Grx-like proteins that expressed well in *E. coli* were tested for activity. Neither Grx-like proteins, CXXC9 and CXXC7 (ROXY1), were found to be active in the HED assay, as determined using two independent batches of purified protein solutions (Table 4.4). Thus it appears that the Grx-like proteins tested do not act as typical of the classical Grxs.

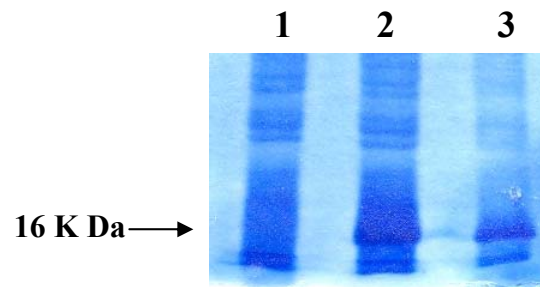


Figure 4.16. Glutaredoxin\_Like Proteins are Soluble in Sarkosyl

The Grx-like protein CXXC9 is shown as being approximately 50% soluble in sarkosyl solution.

1) 4 hour post induced whole cell extract lysed in sarkosyl, 2) soluble fraction from induced cells lysed by sonication, 3) insoluble fraction from induced cells lysed by sonication





Figure 4.17 Purification of CXXC1

Purification procedure performed as described in section 3.2 using sarkosyl cell lysis procedure. SDS Polyacrylamide gel electrophoresis (PAGE) 1) 4 hour post induced whole cell extract, 2) soluble fraction from induced cells lysed by sonication, 3) insoluble fraction from induced cells lysed by sonication 4) fraction 1 from nickel NTA column, 5) fraction 2 from nickel NTA column 6) fraction 3 from nickel NTA column 7) fraction 4 from nickel NTA column 8) fraction 5 from nickel NTA column 9) fraction 6 from nickel NTA column. Fractions 5 and 6 were pooled for use in the HED assay.

Table 4.4 Glutaredoxin -Like Genes Cloned and Activity of Purified Proteins

The activity of CXXC1 activity was calculated from 3 replications from an individual purification. The experiment was repeated on four separate occasions with similar results. Experiments with CXXC7 and CXXC9 were repeated twice with the same results.

Nomenclature Rouhier et al., 2004. 1 U = ( $\Delta$  NADPH) ( volume)/(min)( $\epsilon$ ).

Protein Entry Code	Name	Active Site Sequence	Description	Activity (U)
At5g63030	CXXC1	YCGYC	"Classical Grx active site	153 +/-14
At3g02000	CXXC7	TCCMC	ROXY (Xing et al., 2005)	0
At5g14070	CXXC8	TCCMC	TGA1 Interactor	not tested
At1g28480	CXXC9	GCCMC	TGA1 Interactor	0
At4g15660			Upregulated in tga1/tga4 double mutant (as per microarray analysis)	not tested
At1g03850	CXXS13	GCCLG	TGA1 Interactor	not tested

## Chapter 5: Discussion

In recent years there has been much emphasis on the regulation of defense genes through the TGA family of transcription factors, most notably the factors in clade II (TGA2, TGA5, and TGA6), as they have been shown to bind the SA-inducible *as-1* DNA element found in the *PR-1* promoter (Katagiri et al., 1989; Després et al., 2000). Furthermore, these transcription factors require interaction with an important regulator of the defense responses, NPR1, in order to stimulate transcription of *PR-1* (Després et al., 2000; Rochon et al., 2006).

TGA factors from clade I (TGA1, TGA4) were initially not thought to interact with NPR1 (Després et al., 2000). However, it was later discovered that this interaction required the reduction of two key cysteine residues in clade I factors, not found in clade II factors, and the reduction of these cysteines allowed interaction with NPR1 to occur *in planta* (Després et al., 2003). Additionally, the mutation of these cysteine residues resulted in constitutive interaction of NPR1 and TGA1 both *in planta* and in yeast 2-hybrid assays (Després et al., 2003).

This essential requirement for redox regulation led to the screening of TGA factors in yeast 2-hybrid assays, where TGA2 and TGA7 were initially as the “bait”, in order to identify potential mediators. A family of novel Grx-like proteins was found to interact with both the clade I and clade II TGA factors (Nadamukong et al., 2007; Fobert et al., unpublished). Furthermore, genes encoding several of these Grx-like proteins were also found to be upregulated in microarray experiments of clade I T-DNA

insertion mutants after treatment with SA (Fobert et al., unpublished). These observations led to the question of what role these Grx-like proteins play in the defense response, if any at all.

### **5.1 Glutaredoxin-Like Genes Are Regulated by Salicylic Acid and *Pseudomonas Syringae***

Of the many Grx-like proteins identified in yeast 2-hybrid screens and in microarray analysis four were chosen for qRT-PCR analysis in order to determine if they are regulated in response to SA and *P. syringae*. The four Grx-like protein genes analysed were chosen because the protein encoded were the first ones identified in the laboratory's yeast 2-hybrid screens (*CXXC9*, *CXXC8*, and *CXXS13*), while *CXXS8* was chosen because its transcript levels showed the greatest change in gene expression in microarray experiments comparing wild type to the *tga1-1 tga4-1* mutant. Two of the four Grx-like transcripts were found to be clearly regulated in response to both SA and *P. syringae* (Figures 4.3, 4.4, 4.5, and 4.6). Transcripts of the Grx-like genes *CXXC9* and *CXXS13*, which are the most closely related members of the gene family, were strongly induced in wild type plants after these treatments. While there was some discrepancy in total level of transcript measured between the repetitions and in the timing of peak levels, the overall trend is very clear in that both *CXXS13* and *CXXC9* are induced after treatment of leaves with SA or *P. syringae*. These results are consistent with information found in the public microarray resource Genevestigator ([www.genevestigator.com](http://www.genevestigator.com)), which illustrate that both of these Grx-like transcripts, but not any others studied, are indeed upregulated in response by the treatments performed.

This suggests that these Grx may play a role in the defense response. In contrast to *CXXC9* and *CXXS13*, the expression levels of *CXXS8* are downregulated or unchanged in response to SA and *P. syringae*, suggesting that this Grx-like protein may not play a role in the defense response or, alternatively, that regulation of *CXXS8* may occur at some other level (for example, translation or post-translation).

Transcripts of the Grx-like gene *CXXC8* were found to be inconsistently expressed in response to SA and *P. syringae*. Thus, it is difficult to reach a conclusion about its regulation in response to the two treatments. A recent study sheds some light on the function of this Grx-like protein in the plant, as it appears to be involved in petal formation (Xing and Zachgo, 2008). Mutation of *CXXC8* (AKA ROXY2) does not display a visible phenotype on its own, but in double mutants with its closest relation, *CXXC7* (AKA ROXY1), flowers are completely sterile and deficient of pollen. These two Grxs are also functionally redundant, as *CXXC8* can fully compensate for the loss of *CXXC7*, and have been shown to have overlapping expression in young anthers (Xing and Zachgo, 2008).

## **5.2 Expression of Glutaredoxin-Like genes in Mutants Compromised in Defense Responses Is Complex**

The next question that arises is whether the Grx-like genes are regulated by clade I or clade II TGA factors, or NPR1. Firstly a look at *PR-1* expression reveals some interesting patterns that have not been previously described. In clade I TGA mutants (*tga1-1* or *tga4-1* single mutants and the corresponding double mutant) *PR-1* transcript levels are greater than those measured in wild-type prior to induction with SA or *P. syringae* and remain higher than corresponding wild-type levels in SA-treated

leaves at 4 h and 8 h (Figure 4.1 and 4.2). Leaves infected with *P. syringae* had either decreased transcript levels (compared to wild-type) or returned to wild-type levels at both the 24 h and 48 h time points. These results suggest that the clade I TGA factors may partially repress levels of *PR-I* transcripts in un-induced leaves and in response to SA, but not in response to *P. syringae*. These differences in SA versus *P. syringae* infection may be due to the fact that pathogens elicit a broader response with many interconnecting pathways and thus may have alternative ways of modulating transcript levels of *PR-I*, whereas in the treatment with SA, a smaller branch of the defense response is elicited and there is less interference from intersecting pathways (Beckers and Spoel, 2006).

In the clade II TGA triple mutant (*tga2, tga5, tga6*), transcript levels of *PR-I* are lower than in wild-type prior to treatment with SA, but similar to wild-type levels at 8 h. Consistent with these findings are microarray data showing that *PR-I* transcript levels are no different from wild-type in this mutant (Shearer et al., unpublished). These results suggest that the clade II TGA factors may not activate *PR-I* as reported previously by Zhang et al. (2003), who concluded that transcript levels of *PR-I* in the clade II triple mutant are similar to that of the *npr1* mutants and hence that clade II TGA factors activate transcription of *PR-I*. However, our results indicate that the clade II TGA factors do not play a role in the activation of *PR-I* after treatment with SA, but may weakly activate *PR-I* in the absence of SA. At this time, it is difficult to ascertain why our results differ from those of Zhang et al. (2003). The triple mutant used in this study is the same as the one generated by Zhang et al. (2003) and was provided by Dr. Xin Li. The mutant was analyzed by PCR to confirm the deletion and lack of expression

of TGA2 (Jhadeswar Murmu, unpublished data). It is possible that small difference in growth conditions i.e. light, soil, care and humidity, age of plants (see below), or use of different inducing chemicals (e.g. BTH vs SA) may account for some of the differences.

While the expression of *PR-1* shows a requirement for the clade I TGA factors and NPR1 for repression and activation, respectively, the transcript levels of the Grx-like genes reveal a much more complex mode of expression in the different mutants. Only two Grx-like genes, *CXXS8* and *CXXS13*, had consistently higher levels of transcript in leaves of the clade I mutant compared to wild-type across all four repetitions prior to treatment with SA or *P. syringae*. After treatment with SA or *P. syringae* only *CXXS8* transcript levels remain elevated, compared to wild-type, in all repetitions except for one. These results suggest that *CXXS8* and *CXXS13* may be targets of the clade I TGA factors. As for the Grx-like genes *CXXC8* and *CXXC9*, levels of their transcript in the clade I mutants was variable relative to wild-type, before and after treatment with either SA or *P. syringae*. Accordingly, making inferences into how they may be regulated by the clade I factors is difficult.

The level of transcript for Grx-like genes, *CXXC8*, *CXXS8*, *CXXC9* and *CXXS13* in the *npr1* mutant varied relative to wild-type and it was not possible to determine the role that NPR1 may play in the transcriptional control of these Grx-like genes from these experiments. However, studies done by Nadumukong et al. (2007) indicate that NPR1 does in fact regulate *CXXC9*, as determined by northern blot analysis in the *npr1* mutant background. Similar results have been obtained in the Fobert lab (Patricia Vrinten, unpublished observation). Additional research will be needed to determine

whether these results are dependent on the method of analysis (qRT-PCR vs northern blotting).

Overall each of the four repetitions analyzed showed large differences in relative transcript levels, even if there was a consistent pattern of transcriptional regulation across repetitions. Repetition 3 and R4 often had high levels of transcripts, while R1 was consistently lower in levels of transcript. Growth conditions may account for some of these observed differences; R1 was grown in low light conditions (100  $\mu$ E) and were six weeks old at the time of treatment, while all other repetitions were grown in high light conditions (150  $\mu$ E) and were three weeks old at the time of treatment. Studies have shown that light intensity and induction of *PR-1* are intrinsically linked in both pathogen infected and SA treated plants, presumably due to the failure of adequate HR development (Zeier et al., 2004). The age of the plants may have also influenced the outcome of transcript level and transcriptional control. Age related resistance (AAR) is a phenomenon that occurs separately from the SAR or ISR defense responses but confers similar properties and requires the accumulation of SA in *Arabidopsis* plants ~30-40 days after germination (Kus et al., 2001). Since some of the plants used were several weeks older than what is typically used in our lab, AAR may account for some of the differences observed (Kus et al., 2001).

In general, my results emphasize the importance of repeating gene expression experiments as many times as possible and controlling the conditions as precisely as possible. The latter is often a difficult task to achieve since you cannot always be sure every condition is exactly the same in every repetition; small differences in watering, light, or general care could amount to more or less stress in a plant and thus activate



signaling pathways that interfere with results, as discussed in Clarke and Zhu (2006). Fluctuating environmental conditions may be of particular concern when genetic effects are small. This seems to be likely in the case of clade I TGA control of the Grx-like genes expression since the effect is weak compared to a strong phenotype like the expression of *PR-1*.

### **5.3 Transcript of a Glutaredoxin-Like Gene Is Regulated by Methyl Jasmonic Acid**

The expression of the Grx-like gene *CXXC9* was upregulated in wild type (Col-0) plants after treatment with MeJA (Figure 4.11). This is consistent with a previous report by Nadumkong et al. (2007) and with microarray data from Genevestigator ([www.genevestigator.com](http://www.genevestigator.com)). Since *CXXC9* is upregulated by both SA and JA, it may play a role in SA/JA crosstalk as suggested by Nadumkong et al. (2007). Expression of the Grx-like genes, *CXXS8* did not show and response to MeJA, while *CXXC8* and *CXXS13* were downregulated in one of the two replicates (Figure 4.11). Thus there is not enough evidence to suggest that MeJA plays a role in the transcript accumulation of *CXXC8*. These data may further indicate that the different members of the Grx-like family of proteins have different roles and activities.

### **5.4 Expression of *PDF1.2* Is Repressed in Plants Overexpressing *CXXC9***

To confirm and further the findings of Nadumkong et al (2007) two lines overexpressing *CXXC9* from the CaMV promoter (line #1 *CXXC9* OE, line #2 *CXXC9OE*) were utilized. In wild type leaves an increase in the transcript levels of *PDF1.2* was observed in both R5 and R6 after treatment with meJA as was expected (Figure 4.12). This

increase was not as pronounced in the two lines of CaMV:35SCXXC9. Line #1 CXXC9OE displayed 7-fold lower transcript levels than the wild type at 8 h in R5, whereas no difference was seen between this transgenic line and wild-type in R6 (Figure 4.12). Line #2 CXXC9OE behaved in a more consistent manner and, as shown by Nadamukong et al. (2007), revealed a 5-15-fold decrease in *PDF1.2* transcript levels in both R5 and R6 (Figure 4.12). The most obvious reason for the difference in behavior of line #1 and line #2 are due to expression levels of the *CXXC9* transcript. However, northern blot analysis indicates that the expression levels of line #1 CXXC9OE and line #2 CXXC9OE are similar (Tracy Wignes, unpublished). It has been observed in other studies that correlation between levels of an overexpressed transgene and those of target genes do not always coincide (Friedrich et al., 2001; Lin et al., 2004, Potlakayala, 2007). Transcript level is not the only point at which genes and gene products are regulated. Therefore there may be other factors impeding CXXC9 expression and function that could be affecting the ability of the transgenic lines to repress transcript levels of *PDF1.2*. Measuring levels of CXXC9 protein or activity in the transgenic lines would provide a more accurate comparison of transgene effect. However, this was not possible since we do not have access to CXXC9 antibody with which to perform immunoblots. Similarly, we have not means of monitoring activity of this Grx-like protein (see below).

### **5.5 Tolerance to Oxidative Stress Is Weakened by Overexpression of CXXC9**

Experiments were preformed in order to determine whether overexpression of a Grx-like protein affected the plant's ability to tolerate oxidative stress. Several

replicates were performed using paraquat as the agent of oxidative stress and revealed that line #1 CXXC9OE had poor root growth on paraquat plates compared to wild type roots (Figure 4.13). This difference was determined to be significant in a 2-way ANOVA and Tukey's test. The other line, line #2 CXXC9OE, also had shorter roots than wild-type on paraquat but the difference was not statistically significant (Figure 4.13).

These results were not as expected, since the literature indicates that increasing Grx levels confer enhanced tolerance to oxidative stress in yeast and mammalian cells (Luikenhuis et al., 1998).

Since several of the Grx-like proteins tested did not possess typical oxidoreductase activity (Section 5.4) it is reasonable to assume that these Grx-like proteins are not mediators of oxidative stress as are classical Grx, and therefore would not confer any advantage when overexpressed in transgenic lines. In fact overexpression of CXXC9 had a negative impact on root growth in the presence of paraquat suggesting that increasing levels of CXXC9 may be toxic to the plant, possibly due to altered redox homeostasis.

Once again only 1 line of the two overexpressers proved to be statistically significant for paraquat response, as demonstrated for expression of *PDF1.2* in the preceding section (5.2) but it is a different line in each case that shows the effect. Line #2 CXXC9OE was most effective at repressing expression of *PDF1.2*, while line #1 CXXC9OE shows statistically significant change in root growth. Clearly these experiments should be repeated in order to confirm these findings. Should the same

trends emerge, it could be that the ability to repress *PDF1.2* and tolerance to paraquat are inversely related.

### **5.6 Response to *P. syringae* in *Arabidopsis* with Altered Glutaredoxin-Like Gene Expression Is not Altered**

When looking at the results of the disease test on plants with altered expression of Grx-like proteins there is no statistical difference between wild type and the CXXC9OE in the material treated with  $\text{MgCl}_2$  indicating that the overexpression does not affect basal resistance (Figure 4.14). Furthermore there is also no statistical difference between the wild type and mutants following the avirulent treatment indicating that the SAR response was not stimulated (Figure 4.14). In fact, there was no SAR elicited in the wild-type control plants, as the titres observed in the  $\text{MgCl}_2$  and avirulent pretreatments are not statistically different. Triggering SAR is experimentally difficult and not always observed for various reasons such as: the technically difficult nature of the experiment and infection process and the health and age of the plants. Since my initial trial, this experiment has been repeated with similar results: no effect of the CXXX9 OE lines was observed on either basal resistance or SAR (Heather Shearer, unpublished data). Therefore, there is no evidence to indicate that CXXC9 is involved in the defense pathways. It remains possible that it plays a minor role that could not be resolved under our experimental conditions.

### **5.7 Glutaredoxin-Like Proteins Do not Have Oxidoreductase Activity as Determined by a Standard Glutaredoxin Assay**

In order to determine whether the novel genes encode proteins that have glutaredoxin activity, the HED assay was employed. This assay was chosen because it

is the “gold standard” by which Grx are assayed for activity (Holmgren and Åslund, 1995). Proteins to be tested were expressed in *E. coli* and purified via a C-terminal His tag. A “classical” Grx, CXXC1, and two Grx-like proteins were tested (Table 4.4). The “classical” Grx displayed abundant oxidoreductase activity, while the Grx-like proteins were inactive in the HED assay (Table 4.5).

There are two main explanations for lack of activity: technical or biological. The Grx-like proteins were typically insoluble under the standard purification procedures, while CXXC1 was soluble. This necessitated a modified purification process. Purification of CXXC1 under the modified procedure yielded similar activity levels as obtained by native purification indicating that the purification process was not likely the reason for lack of activity observed with the Grx-like proteins. Another technical reason for lack of activity could be that the epitope tag may have negatively affected activity, either by preventing proper folding or otherwise interfering with the active site. Attempts were made at expressing all of the Grx proteins from an N-terminal HIS-tag vector. However, these attempts were not successful due to problems with poor cell growth and poor protein expression. This may have been due to toxic effects on the cells harboring these vectors. Normally, HIS tags do not interfere with protein folding or activity because they are small, and hence why it was chosen as the epitope tag for these experiments.

The second possibility is that these novel proteins may have sufficiently diverged from classical Grxs and no longer possess typical Grx activity, at least as measured in the HED assay. For example, the novel proteins may not be capable of binding substrates in the HED assay such as glutathione. To date very little research has

been done on the novel family of Grx, with the exception of the work of Xing and Zacho (2008). They indicated, through computer modeling of the Grx-like protein CXXC8, that the active site is similar to the “classical” Grx and can potentially bind to glutathione, but failed to show more formal evidence that it did so. Other studies have also revealed that monothiol Grx in *E.coli* are capable of binding glutathione but do so poorly and at a much reduced rate (Bushweller and Holmgren, 1995).

Several other enzymatic assays have also been routinely utilized to ascertain the activity of oxidoreductases such as the DNTB, insulin reduction, and PTPase assays. These assays were to be attempted, in order to gain further insight into the role of the Grx-like proteins. However, after having left protein analysis to pursue other projects, I could no longer purify the proteins in sufficient amounts for assays. Attempts were made to re-transform the vectors into new cell lines but this did not appear to solve the problem of poor expression. Time constraints became an issue and additional assays were not accomplished. Other studies have also suggested roles for non-classical Grx and the related oxidoreductase protein Trx such as: chaperones, iron or selenium storage proteins, negative regulators of cellular stress responses, and sensitivity to pathogen produced toxins (Rahlfs et al., 2001; Lith et al., 2005; Caldas et al., 2006). Some of these functions require oxidoreductase activity while others do not, such as Trx regulation of the ASK kinase (Saitoh et al., 1998). As described in section 2.6.4 the Trx is bound to the ASK kinase in non-reducing conditions and inhibits activity. However, after oxidative stress the Trx is oxidized and can no longer bind ASK kinase and it in turn is activated.

Thus, although I was not able to obtain evidence that novel *Arabidopsis* Grx display Grx activity by the HED assay, it remains plausible that they possess other, possibly novel, oxidoreductase activities and/or other functions related to classical Grx, PICOT or Trx enzymes that are independent of oxidoreduction.

In conclusion I was able to confirm that the *Grx-like* genes CXXC9, CXXS13, and CXXS8 are regulated in a manner that is consistent with a role in pathogen response. However, only CXXS13 and CXXS8 appear to be regulated by clade I TGA factors. Altering the expression of *Grx-like* genes did not enhance tolerance to oxidative stress, rather it appeared to weaken tolerance nor did the Grx-like proteins exhibit oxidoreductase activity. Perhaps these results are not unexpected given the size and divergence of the Grx-like protein family in *Arabidopsis* one should not be surprised if they too have unique features from one another.

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